Functional biodiversity of *Pseudomonas* species in biofilm communities degrading polycyclic aromatic hydrocarbons

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades einer

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

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eingereicht am: 08.12.2010
mündliche Prüfung (Disputation) am: 02.03.2011

Druckjahr 2011

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeiträge

HECK, MG; ABRAHAM, WR: Multispecies community as an alternative to degrade PAHs (Vortrage). 3rd Joint Conference German Society for Hygiene and Microbiology (DGHM) and Association for General and Applied Microbiology (VAAM), Hannover (2010).

HECK, MG; NEU, T; ABRAHAM, WR: Multispecies community degrades different PAHs (Vortrage). FEMS 3rd Congress of European Microbiologists, Göteborg (2009).

HECK, MG; NEU, T; ABRAHAM, WR: Role of Pseudomonas in multispecies communities degrading Polycyclic Aromatic Hydrocarbons (Poster). Pseudomonas 2009 - XII International Conference, Hannover (2009).

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Abbreviations

ANOVA	Analysis of Variance
BH	Bushnell Haas
CLSM	Confocal Laser scanning microscopy
DDGE	Denaturating Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diaminetetraacetic Acid
egfp	enhanced green fluorescence protein
EPA	Environmental Protection Agency
FAMEs	Fatty Acid Methyl Esthers
GC	Gas Chromatography
GFP	Green Fluorescence Protein
h	Hour
HMW	Hight Molecular Weight
HPLC	High Performance Liquid Chromotoghrapy
LMW	Low Molecular Weight
MeOH	Methanol
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
NAH	Naphthalene
NaOH	Sodium hidroxyde
nm	Nanometer
nMDS	Nonmetric Multidimensional Scaling
PAH	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
PHE	Phenanthrene
rRNA	Ribosomal Ribonucleic Acid
SSCP	Single Stranded Conformation Polymorphism
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
T-RFLP	Terminal Restriction Fragment Length Polymorphism
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
v/ v	Volume per Volume
µg	Microgram
µl	Microliter
µm	Micrometer

1 Introduction

1.1 Sources of Polycyclic Aromatic Hydrocarbons (PAH)

The denominated Polycyclic Aromatic Hydrocarbons (PAHs) comprise a class of organic compounds containing at least two condensed benzene rings. These compounds may also have an hydrogen substituted by a functional group or can contain a heteroatom, such as nitrogen, oxygen or sulphur (Nishioka, 1986 and Smith, 1988). Due to their chemical structure, PAHs are very stable in the environment, have low solubility in water and low vapour pressure, but high melting and boiling points (WHO, 1998). These properties vary depending upon the number of rings and the nature of linkage between them, where more rings result in lower solubility and higher stability of the compound (Figure 1). Depending upon the number of rings, PAHs can be classified either low molecular weight (LMW), those that have up to three aromatic rings, and high molecular weight (HMW), with four or more aromatic rings (Figure 2) (Cerniglia, 1992).

Recalcitrance

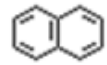
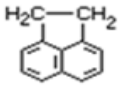
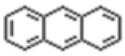
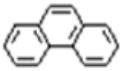
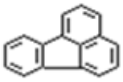
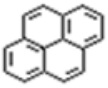
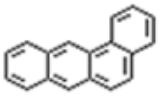
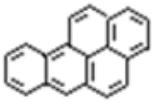
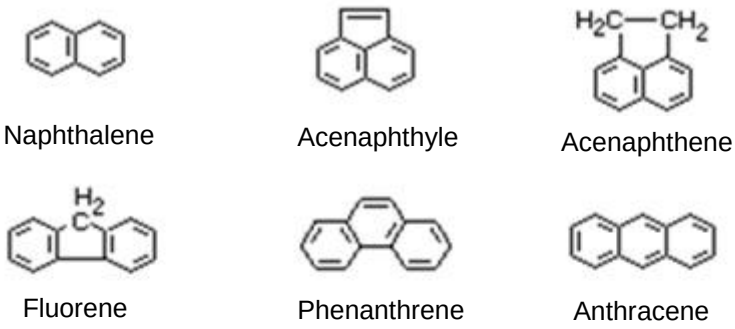
PAH	Molecular Weight	Solubility (mg/l)	Genotoxicity
 Naphthalene	128.2	31.700	---
 Acenaphthene	154.2	3.900	+ Ames
 Anthracene	178.2	0.070	---
 Phenanthrene	178.2	1.300	---
 Fluoranthene	202.3	0.260	Weak carcinogen
 Pyrene	202.3	0.140	± Ames + UDS + SCE
 Benzo(a)anthracene	228.3	0.002	+ Ames + CA + SCE + Carcinogen
 Benzo(a)pyrene	252.3	0.003	+ Ames + CA + UDS + DA + SCE + Carcinogen

Figure 1. Chemical structures, physical and toxicological characteristics of polycyclic aromatic hydrocarbons. The symbols are: (DA) DNA adducts, (SCE) sister chromatid exchange, (CA) chromosomal aberrations, (Ames) *Salmonella typhimurium* reversion assay, (UDS) unscheduled DNA synthesis, (-) not genotoxic (Cerniglia, 1992).

Low Molecular Weight PAHs



High Molecular Weight PAHs

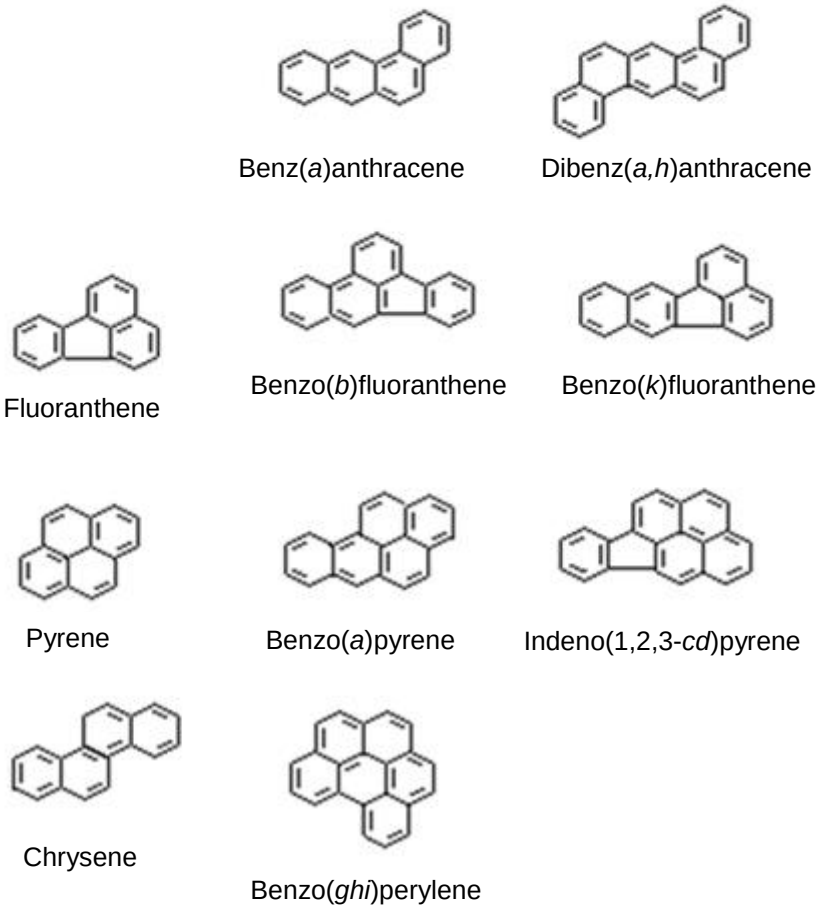


Figure 2. Chemical structure of the 16 PAHs listed as priority pollutants by US EPA.

Aromatic hydrocarbons are produced through natural and anthropogenic processes. The main natural sources of mononuclear and polynuclear aromatic compounds are from petroleum and coal, but they can also arise from biosynthesis of carotenoids, alkaloids, lignin, terpenoids and flavonoids in plants. When these compounds are exposed to high temperatures (for example, volcanic activity and forest fires), long alkyl chains cyclise and with time aromatize. The resultant compound is less substituted the higher the temperature to which it is exposed (Mueller *et al*., 1996). At high temperatures radicals are generated, bind together and then, stabilize through mechanisms as "ring-closing", condensation and dehydrogenation (Zander, 1980). Anthropogenic sources are by far most responsible for environmental contamination, where the largest contribution comes from manufacture of gas and coal tar, wood processing, escape of automobile gasoline and incineration of waste (Harvey, 1991). The production of gas through coal generates residue of coal-tar that used to be stored and disposed directly on to gasification sites. Many of these sites have been abandoned which has led to contamination of soils, sediments and ground water, resulting in extremely contaminated soils containing more than 10 000 ppm (mg kg^{-1}) of PAH (Mueller *et al*., 1996).

Wood processing has also contributed significantly towards PAH contamination. Creosote was largely used to impregnate wood to protect it from rot and worms. Creosote is a high temperature distillated of coal tar comprising 85% of PAH (Mueller *et al*., 1989). The wood was impregnated and allowed to drip dry. In the United States, estimates of 4.5×10^7 kg of creosote usage annually has been reported (Mattraw and Franks, 1986), of which 5-10% is discharged, resulting in PAH environmental contamination (Mueller *et al*., 1996).

There is still one important PAH anthropogenic source that is worthy of considering: accidental spill of crude oil. In this case oil is released directly to the environment compromising several niches. There have been two major cases: the Exxon Valdez oil spill (EVOS) in Prince William Sound, Alaska, USA, where over 250,000 barrels of crude oil (almost 40 million litres) were released on 24 March 1989; and the more recent and the largest offshore spill in history that occurred due to an explosion on 20th April 2010 in Deepwater Horizon drilling in the Gulf of Mexico, USA. In the EVOS the residual oil was largely eliminated in the first few years and is

now widely dispersed, highly weathered or only isolated small pockets of residual contamination remain (Harwell and Gentile, 2006). Sadly, the disaster in the Gulf of Mexico spilled over four million barrels (636 million litres) 16 times more than EVOS, between 20th April to 15th July 2010 when it was finally contained (Camilli *et al*., 2010).

In summary, the sources of PAH in soil are atmospheric deposition, carbonization of plant material and deposition from sewage and waste. According to the World Health Organization (WHO, 1998) the levels of PAH vary depending on the sites: near to industrial areas up to 1g kg^{-1} of each individual PAH can be found; close to automobile exhaust sites 2-5 mg kg^{-1} can be found, and in unpolluted areas, PAH levels are much lower at 5-100 µg kg^{-1} of soil. It is important to note that the contamination of soil is influenced by its composition.

The Canadian Council of Ministers of the Environment created the Canadian Soil Quality Guidelines for Carcinogenic and other PAHs (CCME, 2008), where it was established guidelines with limits of PAH for quality of water and soil:

- potable water (which are the same for all land uses) – benz(a)anthracene: 0.33 mg kg^{-1}; benzo(b+j)fluoranthene: 0.16 mg kg^{-1}; benzo(k)fluoranthene: 0.034 mg kg^{-1}; benzo(g,h,i)perylene: 6.8 mg kg^{-1}; benzo(a)pyrene: 0.37 mg kg^{-1}; chrysene: 2.1 mg kg^{-1}; dibenz(a,h)anthracene: 0.23 mg kg^{-1}; indeno(1,2,3-c,d)pyrene: 2.7 mg kg^{-1};

- soil for agricultural and residential/parkland sites - anthracene: 2.5 mg kg^{-1}; benzo(a)pyrene: 20 mg kg^{-1}; fluoranthene: 50 mg kg^{-1};

- soil for commercial and industrial sites - anthracene: 32 mg kg^{-1}; benzo(a)pyrene: 72 mg kg^{-1}; fluoranthene: 180 mg kg^{-1};

Based on studies of toxicology Jensen and Svendrup (2003) suggested an ecotoxicological soil quality criterion for Denmark of 25 mg kg^{-1} dry weight of soil for the sum of the eight PAHs acenaphthene, fluorene, anthracene, phenanthrene, pyrene, fluoranthene, benz(a)anthracene, and chrysene. The main problem regarding PAH in the environment is that they are recalcitrant, that is they remain for a long time. For example, the three-ring phenanthrene has a half-life in soil of 16 to 126 days, and PAHs with more rings possess even longer half-lifes, such as

benzo(a)pyrene, a five-ring molecule with a half-life of 229 to more than 1,400 days (Shuttleworth and Cerniglia, 1995).

1.2 Degradation of PAH

PAH are products of the incomplete burning of organic compounds such as lignin, an aromatic polymer. Lignin is known to be the second most abundant organic polymer on earth and is a major component of wood (Punnapayak *et al*., 2009). Therefore it is feasible to conclude that degradation of such compounds has evolved by organisms that are in intimate contact or being constant exposed to such compounds. The white rot fungi, for example, are effective in degrading lignin due to the production of lignin-modifying enzymes including lignin peroxidase, manganese peroxidase and laccase. These same fungi are also capable of degrade PAHs due to the similarity between substrates (Bezalel *et al*., 1996). This ability is not just restricted to fungi, but is found in several others microorganisms that have been also exposed to polyaromatic substances through the years and thus have developed mechanisms to utilize them.

Degradation of PAH can also occur by abiotic processes, basically by chemical oxidation and photolysis. The disadvantage of these processes however, is that their products may increase the toxicity of PAHs in eukaryotes (Holt *et al*., 2005). Thus, he biological treatment of PAH contaminated soils should be a more efficient, economic and adaptable choice rather than physicochemical treatment, as it has advantages such as complete degradation of the pollutants, lower cost, greater safety and less disturbance to the environment (Habe and Omori, 2003).

Bacteria can degrade PAH either under aerobic or anaerobic conditions. The aerobic metabolism of PAH has been well studied and described. Metabolic pathways of aromatic compounds are generally divided in to the upper, lower and central carbon pathways. The upper pathway is induced by a specific starting compound; the lower pathway by an intermediary metabolite generated by the upper pathway; and the central carbon pathway is a constitutive, housekeeping pathway (Phale *et al*., 2007).

It is beyond the scope of this introduction to describe the degradation pathways of all the 16 PAHs used in this work, and so it was only included three examples: the pathways of naphthalene, a 2 ring PAH; phenanthrene, a 3 ring PAH and fluoranthene, a 4 ring PAH with some peculiarities in its pathways. In brief, aerobic degradation of PAH starts with incorporation of molecular oxygen into the aromatic nucleus by a multicomponent dioxygenase enzyme system, forming *cis*-dihydrodiol. The resultant compounds are rearomatized by *cis*-diol dehydrogenase to produce dihydroxylated intermediates. These dihydroxylated substrates can be cleaved by dioxygenase *via ortho*- or *meta*-cleavage pathways, leading to central intermediates such protocatechuates and catechols that are further converted to tricarboxilic acid cycle (TCA) intermediates (Habe and Omori, 2003). The proposed catabolic pathways of naphthalene by aerobic bacteria are shown in Figure 3. Naphthalene is the simplest and most soluble PAH, its degradation pathways are used to understand and predict degradation pathways of three or more ring PAHs (Seo *et al*., 2009). When naphthalene is metabolized to salicylic acid, this last compound is typically decarboxylated to catechol, and its ring might suffer *meta*-cleavage (compound 19 in Figure 3) or *ortho*-cleavage (compound 14 in Figure 3). Moreover, salicylic acid may be converted to gentisic acid instead of catechol.

PAHs with three or more rings are degraded in different manner, but in some branches the generated products may lead to the same pathway as that of the naphthalene pathway. This explains why naphthalene has been used as a model compound to investigate degradation of PAH by bacteria. Figure 4 shows the catabolic pathway of phenanthrene. Phenanthrene contains a K-region (Figure 4, structure 1, binding between carbons 9 and 10 in red) that is thought to be responsible for the carcinogenic activity of several PAHs due to the ability of forming epoxides. Thus this compound is used as a model for other PAHs with such regions as benzo(a)pyrene, benz(a)anthracene and chrysene (Seo *et al*., 2009).

Fluoranthene is one of the principal PAHs in the environment (Seo *et al*., 2009). It is degraded by a peculiar pathway due to its five member ring in its structure, and so requires different catabolic steps than those PAHs comprising only with benzenic rings (Figure 4).

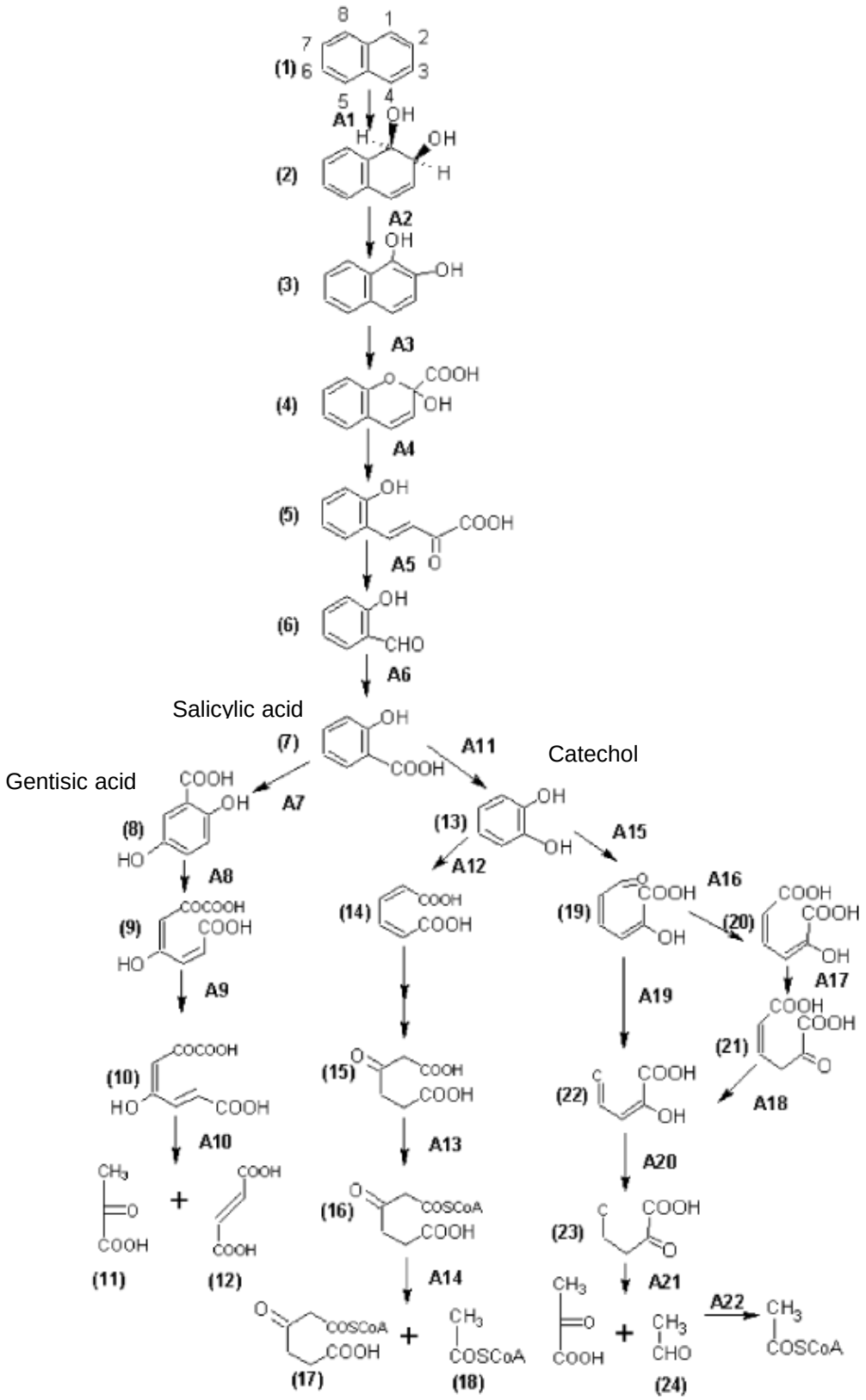


Figure 3. Proposed catabolic pathway of naphthalene by aerobic bacteria (Peng *et al*., 2008).

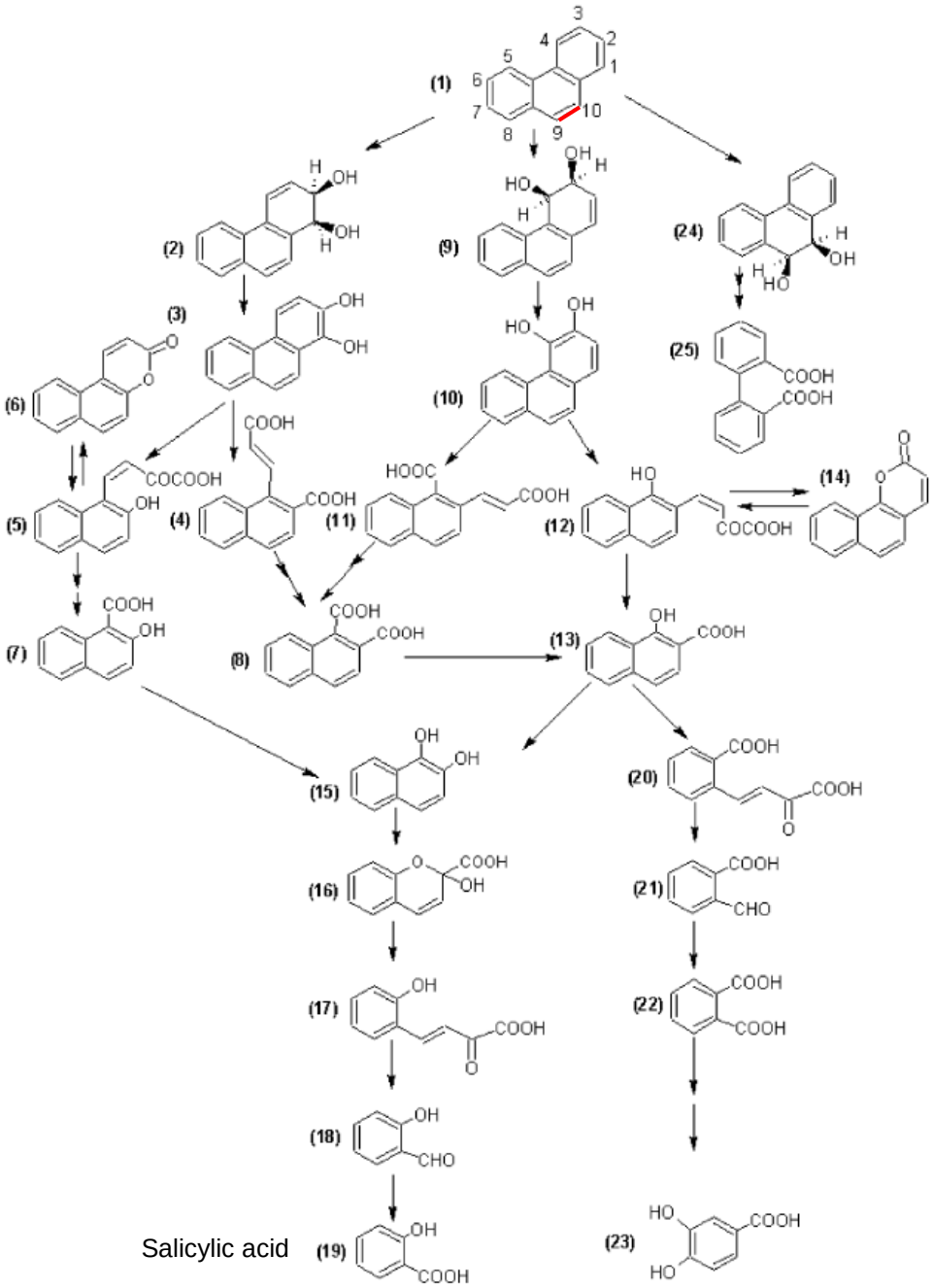


Figure 4. Proposed catabolic pathways of phenanthrene by aerobic bacteria (Peng *et al*., 2008).

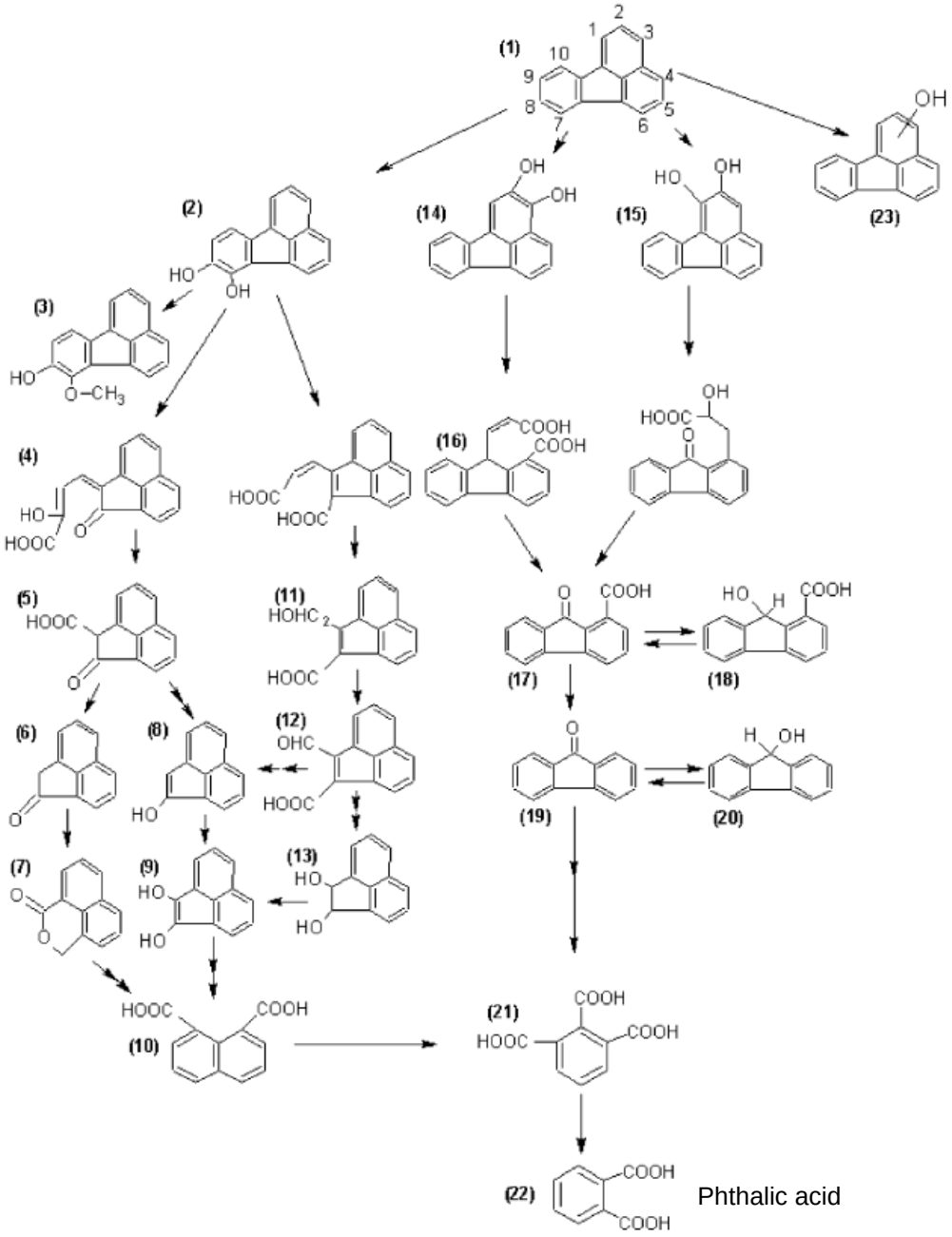


Figure 5. Proposed catabolic pathways of fluoranthene by aerobic bacteria (Peng *et al*., 2008).

Anaerobic degradation of PAHs is not so well studied. The only PAH known to be degraded by pure cultures under anaerobic conditions is naphthalene (Tierney and Young, 2009). Its degradation has been reported under nitrate-reducing and sulfidogenic conditions, either by hydroxylation, carboxylation or methylation as the first step of degradation (Bedessem *et al*., 1997; Safinowski and Meckenstock, 2006; Zhang *et al.*, 2000).

By carboxylation, naphthalene is first methylated with the help of a CO-dehydrogenase, an enzyme associated with acetate oxidation. Then a fumarate moiety is added at the methyl group, producing naphthyl-2-methyl-succinic acid, which is oxidized to naphthyl-2-methylene-succinic acid (Safinowski and Meckenstock, 2006). Several oxidation reactions lead to the formation of 2-naphthoic acid, a central metabolite of anaerobic PAH degradation (McNally *et al*., 1999; Safinowski and Meckenstock, 2006). Zhang *et al*. (2000) identified a lower pathway of naphthalene degradation (Figure 6).

Phenanthrene was found to be metabolized by sulphate-reducing enrichment cultures grown on supplemented phenanthrene (Zhang and Young, 1997) and in sulfidogenic enrichment culture (Davidova *et al.,* 2007) both studies used ^{13}C-labeled bicarbonate. The studies suggest that carboxylation is the mechanism of activation of phenanthrene.

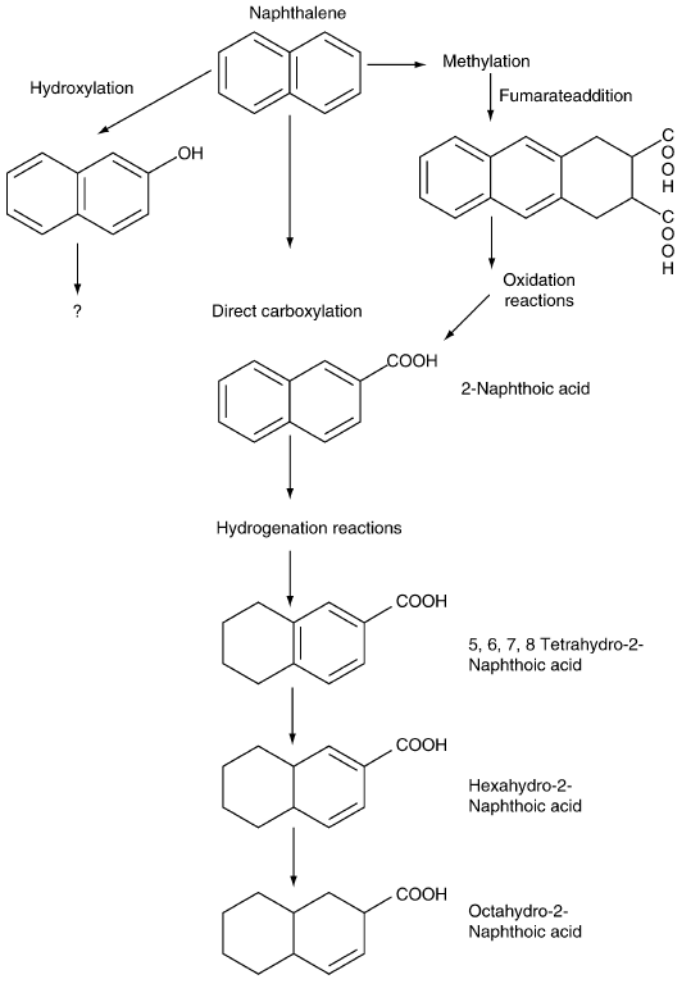


Figure 6. Proposed anaerobic degradation pathway of naphthalene (Tierney and Young, 2009).

1.3 Toxicity of PAH

We can be exposed to PAHs through several sources; wood and coal smoke, burning of fossil fuels, at workplace (coal tar production plants, bitumen and asphalts production plants, municipal trash incinerators, etc.), food and inhalation of tobacco smoke (ASTDR, 1995). PAHs can enter the body through the lungs, by breathing air containing particles of PAHs; drinking water, swallowing food or dust containing PAHs (gastrointestinal tract), or through the skin due to their lipophilicity. PAHs tend to be stored in tissues that contain fat, such as kidney, liver and fat itself. Smaller amounts are also found in the spleen, adrenal glands and ovaries (ASTDR; 1995). In the body, PAH is modified and forms epoxides and diols, that are actually more toxic than the previous compound due to their reactivity. The family of enzymes involved in PAH metabolism is the cytochrom P450 (CYPs), which also contribute to the metabolism of drug, steroids and other xenobiotics. CYPA1 and CYPB1 are monooxygenases that convert inert lipophylic PAH to electrophyles which can be conjugated and excreted from the cell or bind to DNA forming adducts leading to mutation and then cancer (Baird *et al*., 2005).

In 1775, the British surgeon Sir Percival Pott observed that chimney sweepers developed scrotal cancer due to occupational exposure to soot (Boström *et al*., 2002, and Harvey, 1991). This was the first report hypothesing on the carcinogenicity of PAH. One hundred years later in 1875, von Volkman reported elevated incidences of skin cancer in workers in the coal tar industry (Boström *et al.,* 2002). However, it was not until 1930 that Kennaway and Hieger reported the first pure PAH with established molecular structure found to be carcinogenic, dibenz(a,h)anthracene (Harvey, 1991). Moreover, PAHs are not just toxic to humans, they are also toxic to other animals, invertebrates like crustaceans, insects, molluscs, polychaetes, echinoderms, algae and even microorganisms. In fish, for example, it is known that PAH can cause liver diseases, DNA damage, reproductive disorders in females and reduced larva viability, which affect fish populations (Sol *et al.,* 2008). Sverdrup *et al*. (2002) showed that low molecular weight PAH (i.e., naphthalene, acenaphthene, acenaphthylene, anthracene, phenanthrene, fluorene, and the HMW PAHs pyrene and fluoranthene) significantly affect the survival or reproduction of soil-dwelling springtail *Folsomia fimetaria*. The population of arbuscular mycorrhizal fungi

considered essential for the survival of many plants in competitive situation have also been found to be decreased in PAH polluted soils (Cabello, 1997). Table 1 is a summary of genotoxicity and carcinogenicity results for each PAH relevant to the present work.

Table 1. Summary of results of tests for genotoxicity and carcinogenicity for the PAHs relevant to the present work (adapted from WHO, 1998)

Compound	Genotoxicity	Carcinogenicity
Acenaphthene	(?)	(?)
Acenaphthylene	(?)	No studies
Anthracene	-	-
Benz(a)anthracene	+	+
Benzo(b)fluoranthene	+	+
Benzo(k)fluoranthene	+	+
Benzo(ghi)perylene	+	-
Benzo(a)pyrene	+	+
Chrysene	+	+
Dibenz(a,h)anthracene	+	+
Fluoranthene	+	(+)
Fluorene	-	-
Indeno(1,2,3-*cd)*pyrene	+	+
Naphthalene	-	(?)
Phenanthrene	(?)	(?)
Pyrene	(?)	(?)

+, positive; -, negative; ?, questionable
Parentheses, result derived from small database

The United States Environmental Protection Agency (USEPA) and the European Union have listed 16 PAHs as priority pollutants based on their toxic, mutagenic and carcinogenic properties: naphthalene, acenaphthene, acenaphtylene, anthracene, phenanthrene, fluorene, fluoranthene, benz(a)anthracene, chrysene, pyrene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenz(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-*cd*)pyrene (Rigging and Strup, 1984) (Figure 2). Those are the PAHs chosen for the present work.

In summary, the main concerns regarding PAHs is that they are ubiquitous, toxic and persistent in the environment.

1.4 Research approaches used to date

Culture-based approaches have been extensively used to study PAH degradation and many bacteria species have been found to be capable in doing it. The most representative genera responsible for PAH degradation are: *Aeromonas, Acidovorax*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter, Bacillus*, *Brevibacterium*, *Comamonas*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Stenotrophomonas*, *Streptomyces*, and *Xanthomonas* (Doyle *et al*., 2008). These monoculture studies have been extremely valuable because they have unravelled different pathways which microorganisms use to metabolize different compounds in pure cultures. However, in the environment bacteria are seldom in pure culture, they interact with many other organisms. In soil, for example, they cannot always swim freely as planktonic cells, like they do in liquid cultures in the laboratory, they attach to surfaces and live in communities. The aim of several more recent projects is not to isolate one single strain, but identify consortia of many species able to deal with complex mixtures such oil and diesel fuel. Kanaly *et al*. (2000) used non-contaminated soil and added crude oil to stimulate the growth of degraders. After 2 years, they obtained a stable consortium which degraded jet fuel and benzo(a)pyrene labelled with ^{14}C. The community was analyzed by a culture-independent method called denaturating gradient gel electrophoresis (DGGE). This method separates fragments of DNA which differ in their sequence but not size, where separation is based on the decreased electrophoretic mobility of a partially melted doublestranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants - a mixture of urea and formamide (Muyzer and Smalla, 1998). The characterized community was composed of 10 members, from which 9 were Gram negative *Proteobacteria*. The most representative bacteria from this consortium were species already known as degraders: *Sphingomonas*, *Mycobacterium* and *Alcaligenes*. This consortium could mineralize 65-75% of 10 mg of added benzo(a)pyrene. This is a rare study where non polluted soil was studied. A different study, conducted by Eriksson and colleagues (*Eriksson et al*., 2000), assessed PAH-contaminated soil from an old gas work site inoculating it in a microcosm. The 5 bacteria isolated from it belonged all to the *Pseudomonas* genus. The authors concluded that when hydrocarbons are added to soil, it remains as an

available fraction that is consumed in a few days, unlike the sorbed compounds from previous contamination, which remained unaffected by degradation and microbial attack. In another study with soil contaminated from an asphalt and tar production, the soil was fertilized for 4 years before isolation of microorganisms, from which 6 belong to the *Mycobacterium* genus and one was an *Arthrobacter* species (Johnsen *et al*., 2007). It was observed that the degraders from the polluted soil showed high survival rates in a control microcosm without PAH and were still culturable after 16 weeks of incubation, demonstrating the greater fitness of soil-adapted cells compared to laboratory strains introduced into soil microcosms showing usually fast decline. A comparison between isolates of a consortium from soil of a petrochemical complex and members of the community identified through a culture-independent method, DGGE (Molina *et al*., 2009), showed that the two methods identify one different member of the community. Although all members belong to the *Gammaproteobacteria* phylum, the isolates were *Enterobacter* sp. and *Pseudomonas* sp., while the sequences obtained from the DGGE were *Pseudomonas* sp. and *Stenotrophomonas* sp. In a similar fashion, Hilyard and co-workers (Hilyard *et al*., 2008) compared isolates with sequences obtained also from DGGE and, although similarities were observed, not all sequences correlated with the isolates. It is clear then, that culture-independent methods are a robust tool to identify bacteria from soil that are usually underestimated or even not characterized using classical isolation methods, probably because they do not fit to laboratory conditions or, conversely, bacteria that are not as representative in the soil gain competitive advantages under laboratory conditions.

While the studies described above used DGGE to fingerprint the bacterial community, there are a suit of other methods that are at our disposal which employ polymerase chain reaction (PCR) amplification of genetic markers using universal primers capable of amplifying the target genes from a wide variety of different organisms. Nocker *et al.* (2007) reviewed this suite of molecular DNA-based approaches that rely either on direct cloning and sequencing of DNA fragments (shotgun cloning) or rely on prior amplification of target sequences by use of the polymerase chain reaction (PCR). The pool of PCR products can then be either cloned and sequenced or can be subjected to a variety of genetic profiling methods, including: amplified ribosomal DNA restriction analysis (ARDRA), automated

ribosomal intergenic spacer analysis (ARISA), terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), and denaturing high-performance liquid chromatography (D-HPLC). In their review, they critically compared these methods commonly used to study of microbial diversity from different environments. Smalla and colleagues (Smalla *et al*., 2007) compared the pattern of different soil communities by DGGE, SSCP and T-RFLP and concluded that all three fingerprinting techniques provided similar, but not equal results, but the reason for this is probably due to the primers used, which comprised different variable regions. However, Hori *et al*. (2006) performed a comparison between DGGE and SSCP using primers amplifying the same variable regions V3-4. They demonstrated that SSCP was superior in detecting the dynamics of the analyzed community over DGGE, providing more and sharper bands and capable of detecting subtle differences within the community, undetected by DGGE.

The advantage of using mixed microbial communities instead of pure cultures to promote degradation of complex compounds and mixtures is that different bacteria possess different enzymatic machinery to degrade diverse compounds. As a result one strain might be able to degrade one intermediate generated as a dead end from another strain, promoting a complete mineralization of the substance. So, if we start working with such mixed microbial communities for this purpose, then we also need to be able to assess/elucidate their diversity/abundance throughout the degradation process. Thus, having a suite of appropriate culture-independent methods at our disposal is essential.

A study by Pawelczyk (2007) showed degradation of 4-chlorosalicylate by a community of *Pseudomonas reinekei* MT1, *Achromobacter spanius* MT3, *Pseudomonas veronii* MT4 and *Wautersiella* falsenii MT2. MT1 and MT3 were first inoculated in a chemostate, they reduced the level of 4-chlorosalicylate from 5 mM to 0.04 mM, then MT4 and MT2 were added to the culture but did not change the amount of the substrate, instead the amount of protoanemonin and cis-dienlactone, dead-end metabolites of the 4-chlorosalicylate degradation pathway by strain MT1 decreased, showing that the addition of the other strains was necessary to continue the degradation of the generated products. Moreover it was shown by Pelz (Pelz *et*

al., 1999) that strain MT2 is a “necrotiser” of the consortium, this strain doesn’t actually degrade the substrate, but survives by degrading the substrate produced by dying cells, further contributing to the stability of the consortium.

The present study was based on the hypothesis that communities are more effective than pure culture for degradation studies. Different soil and sediment samples were taken to study community behaviour and PAH degradation. It employed classical microbiological methods for isolation of bacteria, those were used to create artificial communities; and also soil and sediment were used straight from the environment in microcosm experiments where communities were selected without previous enrichments and were analyzed by culture-independent methods (SSCP and T-RFLP) to identify members of these communities and follow their dynamics during PAH degradation. The great challenge of this project was to not use liquid cultures, as is done in the vast majority of studies, instead microcosms were constructed with soil as inoculum and PAH were added as crystals attached to a solid surface, where bacteria should bind and form a biofilm.

The specific questions addressed in this project were:

1. Is it possible to select communities directly from the environment (without isolation of strains) capable of colonizing and degrading PAH crystals?
2. How do these communities behave over time? Do their members remain over time?
3. Is it possible to isolate the members of these selected communities?
4. Are artificial communities (those constructed with isolates) as efficient as the communities selected from the environment (without previous isolation of strains)?
5. Would different PAH substrates select for different genera of a community?
6. What is the role of the genus *Pseudomonas* concerning degradation of PAHs in environmental communities?

2 Materials and Methods

2.1 Experiment design

In the present work sediment and soil samples were used to perform microcosm experiments in which degradation of PAHs were analyzed and bacterial communities were characterized by Single Strand Conformation Polymorphism (SSCP). Moreover bacterial strains were isolated and utilized in additional microcosm experiments (see Figure 7 – below).

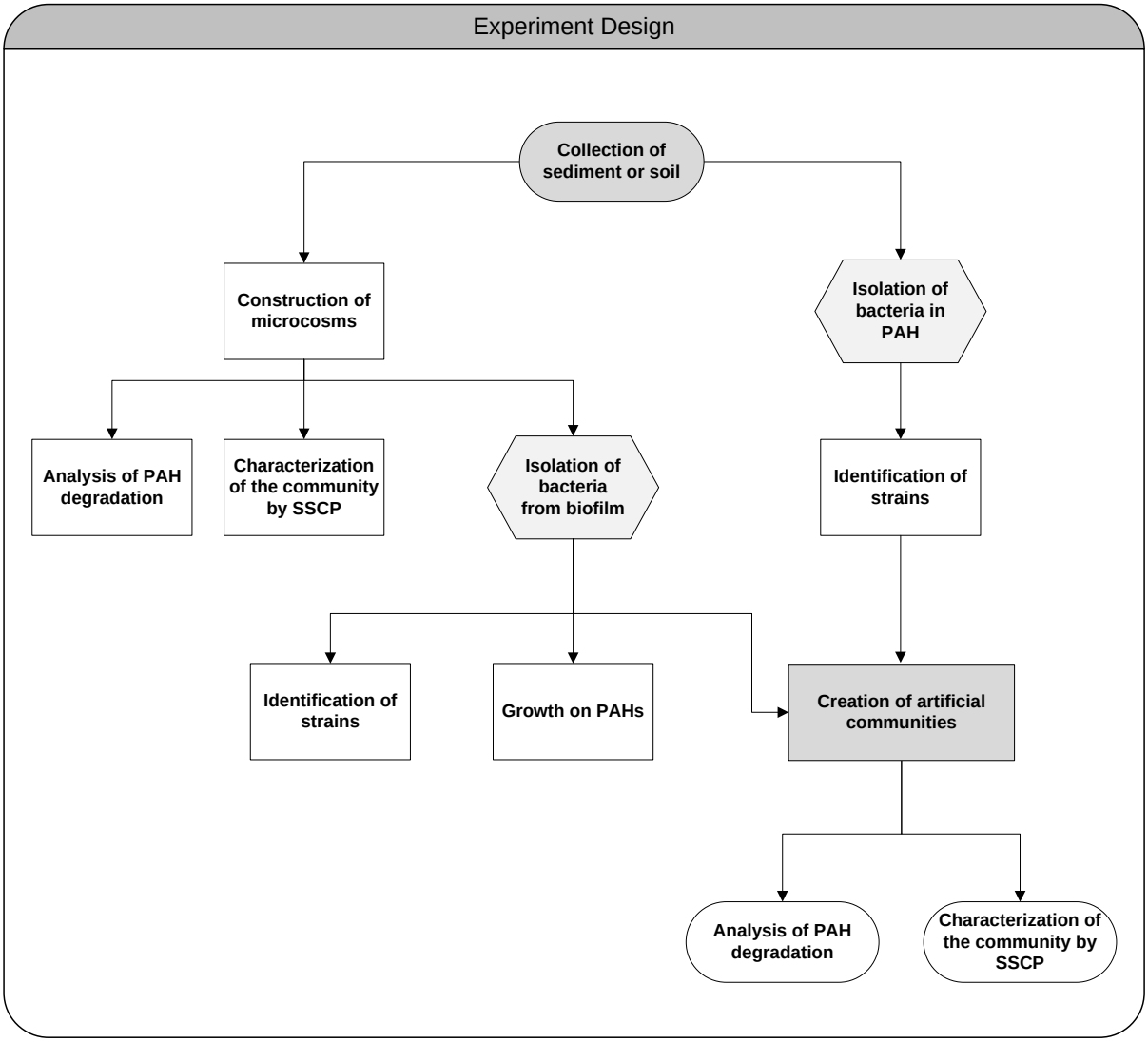


Figure 7. Experiment design of the present study.

2.1.1 Collection of sediments and soil for microcosm experiments and bacterial isolation

Sediments and soil samples were collected at three different locations: Oderteich, Waldau and Abtsdorf in central and eastern Germany, as shown in the Figure 8, between May of 2008 and October of 2009.

Figure 8. Location where sediment and soil samples were collected. (A) Oderteich, (B) Abtsdorf and (C) Waldau (adapted from WT, 2010).

In Oderteich it was taken sediment from a small stream flowing into Oderteich reservoir, in the Harz Mountains, Germany (as previously used in the work of Macedo *et al*., 2007).

In Waldau it was taken sediment from a small creek used for dumping the waste of lignite mining and factory of briquette (coal) (personal communication from Professor Walther Gläser), in east Germany, Thuringia. At this location samples were collected at two different time points, the first in May 2008 (Spring) and another in October 2009 (Autumn). In both cases samples were taken from different sites along the creek. In May, 2008 the sites were named as number 1 to 9, 1 being the closest to a bridge used as a reference point and 9 the most distant from the bridge; in October, 2009 the samples were named as letters A, B, C, D, E and F; A being the closest to the bridge, E the most distant from the bridge, and F was the sample taken in the opposite site of the bridge.

Abtsdorf is a PCB-polluted site located within a former military base north of Wittenberg (Germany), This site and detailed physical and chemical characteristics of the soil were reported previously (see Nogales *et al*., 1999). At this location 4 sites were sampled, named as B, C, 54 and Clean.

The samples were collected in plastic bags, stored at 4°C and used within 6 weeks. The only except being microcosm experiment 6, where the sediment was stored at 4°C for seven months before use (more details are provided latter). The collected samples were used to perform microcosm experiments (described in section 2.1.2, table 3) and some were further used for bacteria isolation (Table 2 – below).

Table 2. Sample imformation

Location	Site	Date	Microcosm experiments conducted with the sample	Source for bacterial isolation
Oderteich	1	May 2008	Yes	Soil
Oderteich	2	May 2008	Yes	Soil
Waldau	3	May 2008	Yes	Soil Biofilm
Waldau	7	May 2008	Yes	n.a.
Abtsdorf	B	October 2008	Yes	Biofilm
Abtsdorf	C	October 2008	Yes	Biofilm
Abtsdorf	54	October 2008	Yes	n.a.
Abtsdorf	Clean	October 2008	Yes	n.a.
Waldau	A	October 2009	Yes	n.a.
Waldau	B	October 2009	Yes	n.a.
Waldau	C	October 2009	Yes	Enrichment experiment
Waldau	D	October 2009	Yes	n.a.
Waldau	E	October 2009	Yes	n.a.
Waldau	F	October 2009	Yes	n.a.

Yes = samples used in microcosm experiments.

n.a.= bacteria were not isolated or not used in experiments of the present work.

2.1.2 General microcosm set-up

Soils or sediments were placed into glass vessels in sufficient amount to cover the bottom, sterile water or minimum medium was added to form a liquid layer of about 2 cm over the sediment / soil. PAH crystals were added on Permanox® slides (Nunc, Germany) (a polyester polymer inert to organic solvents) by dropping a solution of PAH dissolved in dichloromethane and allowing it to dry for at least 1 h. In each microcosm slides with PAH crystals were added into the microcosm facing down into the water (Figure 9).

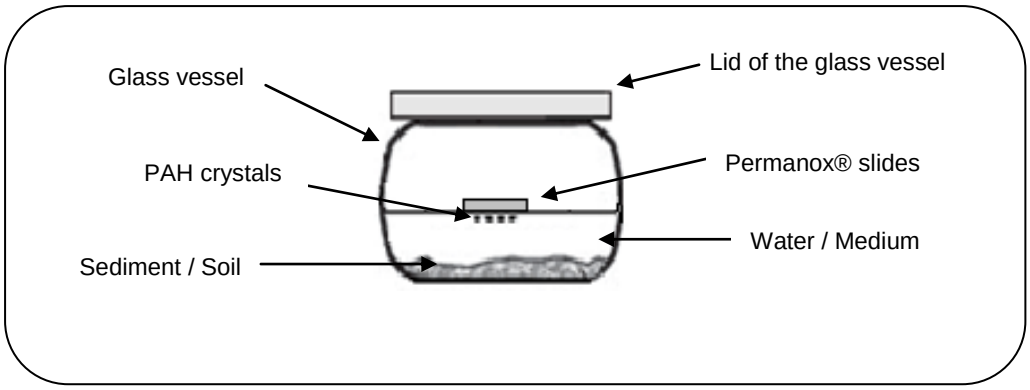


Figure 9. Scheme of the microcosm set-up.

In microcosm experiment 1, the PAH crystals were dried from 25 µl of a solution containing 1.5-2.0 mg ml^{-1} of each of the 16 PAHs: naphthalene, acenaphthene, acenaphtylene, anthracene, phenanthrene, fluorene, fluoranthene, benz(a)anthracene, chrysene, pyrene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenz(a,h)anthracene, benzo(g,h,i)perylene and indeno(1,2,3-*cd*)pyrene (Figure 2). In the further microcosm experiments, the volume of the PAH solution was reduced to 15 µl because it was observed that smaller volume led to less differences in the crystal amount caused by evaporation of the solvent in the pipette tip. The concentration of the PAH solution was kept the same (1.5-2.0 mg ml^{-1} of each of the 16 PAHs) in microcosm experiments 2 and 3, but in the microcosm experiments 4 and 5 it was decreased to 0.5 mg ml^{-1} to reduce further dilution procedures. However, in microcosm experiment 6, a solution containing 2.0 mg ml^{-1} of PAHs was used. In microcosm 5 it was used a solution containing 6 PAHs: naphthalene, anthracene, fluoranthene, phenanthrene, pyrene

and chrysene; whereas in microcosm experiment 6 the PAH solution was made with only 4 PAHs: anthracene, fluoranthene, phenanthrene and pyrene This change was made in order to simplify further analysis and observe carbon flow using each of these PAH labelled with ^{13}C and measuring incorporation of them in the fatty acids of the bacteria. Table 3 summarizes the set-up of each microcosm experiment. For every set of experimental microcosms, autoclaved soil was used as a blank to compare the loss of PAH due to abiotic processes.

Sampling of the microcosms was performed at different time points depending upon the experiment, but usually up to 60 days of inoculation. DNA was extracted from the samples in order to characterize the community by SSCP and the residual amount of PAH was measured by HPLC. Some time-point samples were also used for confocal and field emission scanning electron microscopy.

Table 3. Description of the microcosm experiments.

Microcosm Experiment	Samples used	PAHs used	Concentration of PAH used	Volume of solution used (drop)
1	Waldau 3 Oderteich 1 Waldau 7 Oderteich 2	16 PAHs	1.5-2.0 mg ml^{-1}	25 µl
2	Abtsdorf B Abtsdorf C Abtsdorf 54 Abtsdorf Clean	16 PAHs	1.5-2.0 mg ml^{-1}	15 µl
3	Isolates from Waldau 3 - Soil - Biofilm	16 PAHs	1.5-2.0 mg ml^{-1}	15 µl
4	Isolates - Waldau 3 - Abtsdorf B - Abtsdorf C - Additional isolates (SP)	16 PAHs	0.5 mg ml^{-1}	15 µl
5	Waldau A Waldau B Waldau C Waldau D Waldau E Waldau F	6 PAHs: naphthalene anthracene, fluoranthene, phenanthrene, pyrene and chrysene	0.5 mg ml^{-1}	15 µl
6	Sediment Waldau C - Sediment stored at 4°C for 7 months - Sediment enriched for 7 months due to used in microcosm experiment	4 PAHs: anthracene, fluoranthene, phenanthrene and pyrene	2.0 mg ml^{-1}	15 µl

2.1.3 Set-up of the 6 specific microcosm experiments

2.1.3.1 Microcosm experiment 1: Waldau, Oderteich

In microcosm 1, a single large glass container (with dimensions of about 29 x 17 cm) was used, where all slides with PAH were placed together (see Figure 10).

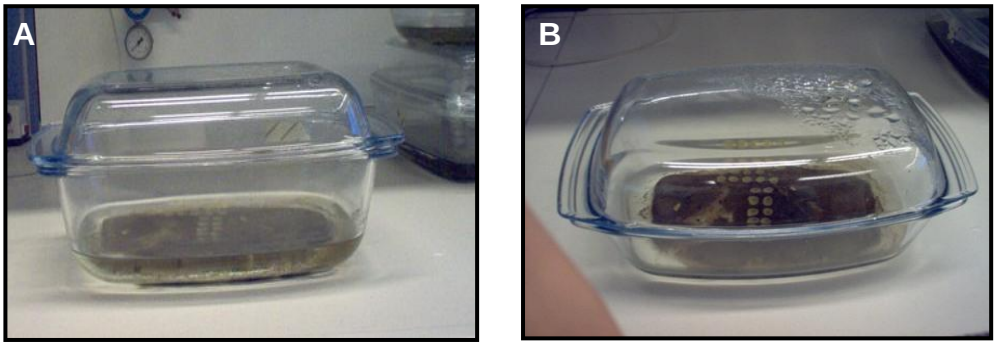


Figure 10. Glass container used in microcosm experiment 1.

This experiment was divided in two parts:

- Part 1 – Sediments incubated with slides containing the 16 PAHs.
- Part 2 - Sediments incubated with slides containing single crystals of PAH.

2.1.3.1.1 Microcosm experiment 1 - Part 1: Waldau, Oderteich

Samples from Waldau, site 3 (W3) and site 7 (W7); and Oderteich site 1 (Od1) and 2 (Od2) were used in the first microcosms constructed in this work. Sterile water was the liquid layer between soil and slides. Samples were taken at 1, 7, 14, 21, 28, 35, 42, 90 and 180 days.

2.1.3.1.2 Microcosm experiment 1 – Part 2: W3 Single crystals

Observing the development of these microcosms communities another question was raised: do the communities depend on the substrate, or will they be the same if we use single PAHs? To answer this question slides containing single crystals of each PAH were added to the microcosm W3 already enriched for 5 months. After 60 days the slides were analysed for their community structure. A matrix was constructed for statistical analysis with data from SSCP gel base on presence or absence of bands.

2.1.3.2 Microcosm experiment 2: Abtsdorf

Soil samples collected in Abtsdorf labelled as B, C, 54 and Clean were used to construct these microcosms. The liquid layer was also formed with sterile water. Samples were taken after 1, 10, 21 and 60 days.

2.1.3.3 Microcosm experiment 3: Isolates from Waldau 3

In this experiment three different microcosms were constructed with isolates from Waldau site 3 (W3):

- Isolates from sediment: comprising 11 isolates
- Isolates from the biofilm: comprising 16 isolates
- All the above, isolates from sediment and from the biofilm: 27 isolates

The description of the isolates and its sources are listed in Table 3 (section 3.2.1)

2.1.3.4 Microcosm experiment 4: Isolates from Waldau 3, Abtsdorf B and C and 5 other isolates

In this experiment five different microcosms were constructed, 2 using different media without a carbon source, and 3 supplemented Bushnell Haas (BH) medium:

1. Bushnell Haas (BH) medium
2. M9
3. BH supplemented with 0.01g l^{-1} Glucose
4. BH supplemented with 0.013 g l^{-1} yeast extract
5. BH supplemented with 0.02 g l^{-1} peptone

In total 52 isolates were selected (Figures 25 and 26). Due to the large number of available isolates, if more than one isolate presented 100% similarity on the sequence of 16S rRNA, just one was selected to be used in this experiment. The other criteria of selection was source of isolation and respective capability of the community in degrading PAHs, confirmed in previous microcosms experiments; like this it was utilized isolates from W3, sediment and biofilm; from Abtsdorf B and C isolated from the biofilm, and 5 isolates from sediment of a polluted river Rio Pinheiros, São Paulo, Brazil. These last five were selected because they were isolated using agar plates with PAH as the only carbon source: *Pseudomonas* sp. (3 isolates), *Ochrobactrum* sp. (1 isolate) and *Kaistia* sp. (1 isolate). All microcosms were inoculated with 100 µl of each bacteria culture adjusted at an optical density of 0.2 at 580 nm (OD_{580}).

2.1.3.5 Microcosm experiment 5: Waldau 2009 - New sediment collection

After concluding that the Waldau site had the best degraders in this study, new samples were collected at this location in October, 2009. The sites where the samples were taken were named as A, B, C, D, E and F. Microcosms were constructed to check whether the bacteria were still active, as they were collected in autumn (October 2009) instead of spring (May 2008). The same name as the sampling sites were given to the respective microcosms, A, B, C, D, E and F. In this microcosm Bushnell Haas medium was used as the liquid layer and samples were taken every 10 days up to 60 days.

2.1.3.6 Microcosm experiment 6: Transfer of slides to sterile microcosms

This last set of microcosm experiments was divided in two parts:

- Part 1 – microcosms were constructed using soil from Waldau C stored at 4°C ($Wal.C_s$) for almost seven months, and soil already used in Microcosm 5, Waldau C kept in a microcosm for almost seven months after the first contact with the slides with PAH crystals ($Wal.C_{enriched}$). Slides without PAH were used as controls.

- Part 2 - After 10 days, slides from the microcosms (Part 1) containing 2 drops of PAH colonized by bacteria, were transferred to new microcosms with sterile Bushnell Hass medium in which new slides with PAH crystals (anthracene, fluoranthene, phenanthrene and pyrene) were added.

In summary, there were microcosms Wal.C_s and Wal.$C_{enriched}$ inoculated with sediment; and t-Wal.C_s and t-Wal.$C_{enriched}$ inoculated with slides transferred from the respective microcosms (Figure 11). A semi-quantitative approach was used to analyse the SSCP gels, grades from 1 (faint thin bands) to 5 (strong wide bands) were given based on the width and intensity of the bands.

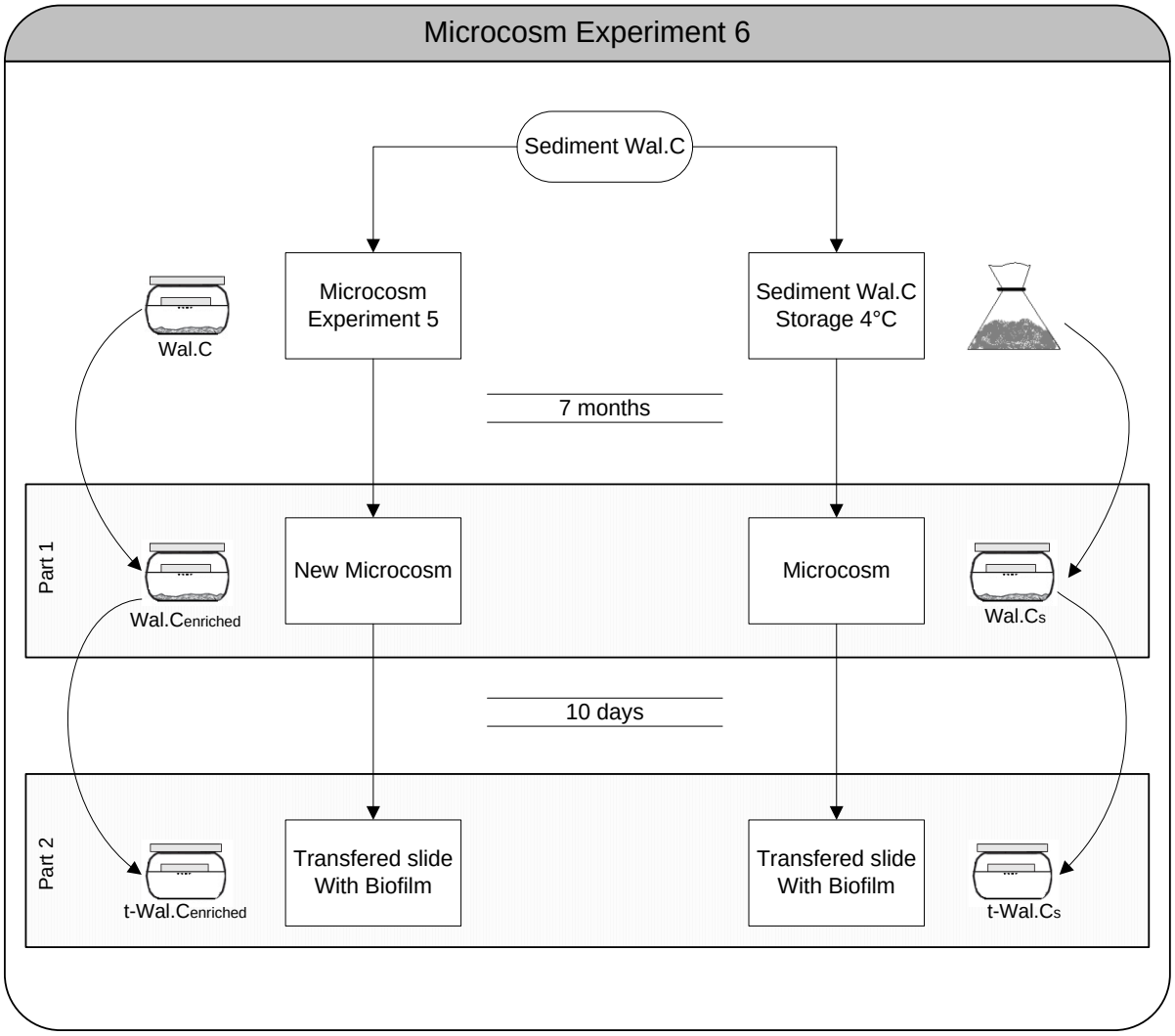


Figure 11. Experiment design of microcosm experiment 6.

2.1.4 Bacterial enrichment of Waldau sediment

The last samples collected in Waldau (2009) were use to perform classical enrichment experiments to compare the community obtained from this experiment and those from the microcosm set-up. Ten grams of sediment were added to 90 ml of Bushnell Haas medium containing 5 mg of a mixture of 4 PAHs: anthracene, fluoranthene, phenanthrene and pyrene. After 10 days, 1 ml was transferred to 100 ml of fresh Bushnell Haas medium containing the same PAHs mixture, after additional 10 days the procedure was repeated and after 10 more days a serial dilution in PBS buffer was made with this last culture and bacteria were isolated in media R2A diluted 10 times. After growth, colonies were picked and transferred to new plates until pure cultures were obtained (Figure 12).

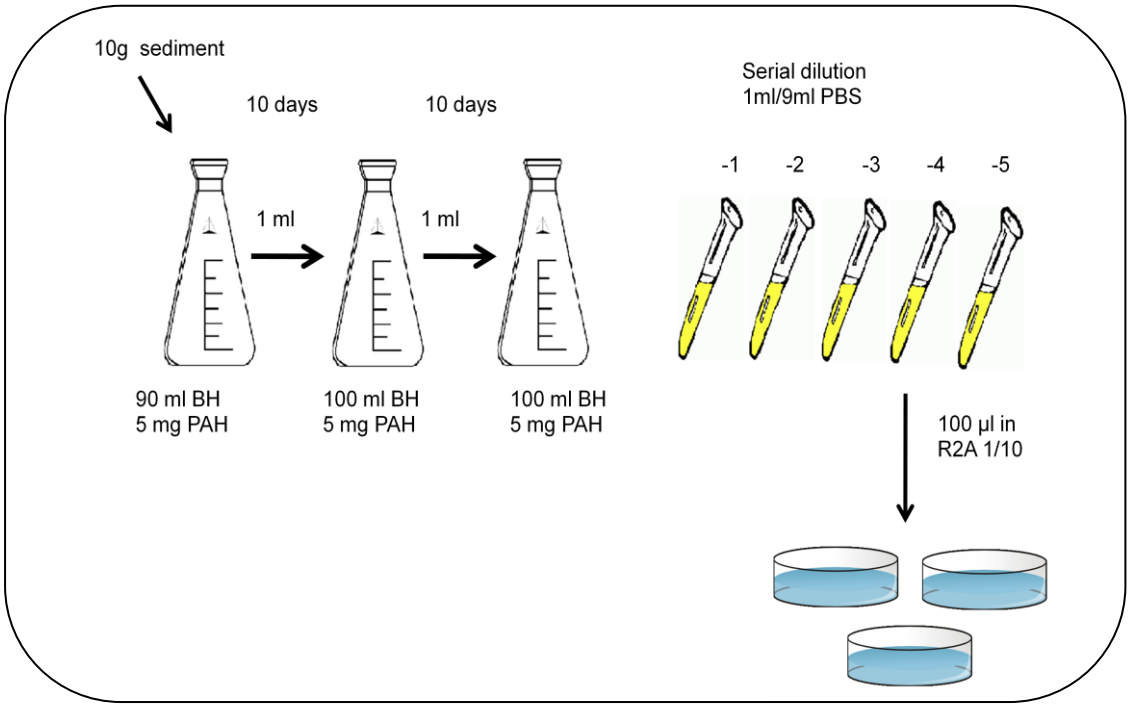


Figure 12. Bacterial enrichment of Waldau sediment

2.1.5 Bacterial isolation

Bacteria were isolated from the soil through serial dilutions in PBS buffer. One hundred microliters of the dilution were plated in medium M9 with Trace element

solution and 0.5 mg of one PAH as the carbon source. The PAHs used as carbon sources were anthracene, fluoranthene, phenanthrene and pyrene. They were dissolved in acetone, put into the medium and allowed to dry for at least one hour.

To isolate bacteria from the biofilm formed on the PAH crystal, slides containing the biofilm were scratched with sterile swabs wetted with TE buffer, and used to seed plates containing medium R2A and LB, both diluted 10 times.

In both cases colonies were picked and transferred to new plates until pure cultures were obtained.

2.1.6 Stock cultures

For stock cultures a colony from the pure bacterial culture was added to 750µl sterile medium (LB or R2A, depending on the isolate) in a 2 ml cryo-vial and incubated for 24 hours at room temperature. After growth, 500 µl sterile glycerine was added, the vial was vortexed and then frozen at -20°C.

2.1.7 Liquid cultures for inoculation

Bacterial strains from frozen stock cultures were streaked on to R2A or LB agar plates and incubated at room temperature until colony formation was visible. Single colonies were used as inoculum for liquid cultures.

2.1.8 DNA Extraction

DNA was extracted with different approaches:

- Biomass was scratched from the slides with a sterile swab, the swab was placed into a 1.5 ml tube with 250 µl of TE buffer and kept at 96°C for 15 minutes in a heating block. The solution containing the DNA was kept at -20°C until further analysis.

- Permanox® slides containing the biofilm growth on PAH crystals were cut and placed into a column provided in the commercially available Fast-DNA-Spin-Kit® for soil (Q-Biogen, MP Biomedicals, Heidelberg) and DNA was extracted following the manufacturer's instructions. DNA was kept at -20°C until further analysis.

- DNA from bacterial isolates was extracted by adding one loop of the isolate into 50µl of TE buffer and kept at 96°C for 15 minutes in a heating block. This DNA was used for 16S rRNA PCR and in case of a negative result DNA extraction was repeated with Spin-Kit® for soil.

2.1.9 PAH extraction

The Permanox slides from the microcosms were taken, cut in a way to have only one drop of the PAH mixture and placed inside of an extraction flask, in which was added up to 2 ml of dichloromethane and agitated for 2 minutes. The extracted slides were removed from the flask, rinsed with dichloromethane and discharged. The PAH solution was concentrated to 100-200 µl, quantitatively transferred to a 1ml volumetric flask and the volume was completed with acetonitrile. The samples were transferred to 1.5 ml glass vials and further dilutions were made accordingly to the detection range of the HPLC detector.

2.1.10 Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC is a technique for separation of molecules based on the capacity of hydrophobic molecules to adsorb onto a hydrophobic solid support, the column, in a polar mobile phase. Decreasing the mobile phase polarity by using organic solvents the hydrophobic interaction between the solute and the solid support is reduced resulting in de-sorption. The more hydrophobic the molecule the more avidly it will adsorb onto the solid support, thus higher concentration of organic solvents will be

required to promote de-sorption. The detector records a chromatogram as a function of retention time, wavelength and absorption.

The analysis of the amount of PAH was done in a Waters Alliance 2695 Separation Module HPLC equipped with a Pursuit PAH column, particle size 3 µm; length 100 mm and internal diameter of 4.6 mm (Varian, Middelburg, Netherlands). The mobile phase was water:acetonitrile at a flow rate of 1.0 ml min^{-1}. The concentration of acetonitrile was initially 40% (1 minute) then it was increased to 100% in 40 minutes and kept like this until 45 minutes. The column effluent was monitored from 210 to 400 nm by a Photodiode-Array-Detector and a Scanning Fluorescence Detector. Table 2 shows the detection program of the fluorescence detector. The injection volume was 10 µl. Data were analyzed by using the Empower 2 software (Waters, Eschborn/Germany).

After appropriate dilutions, the amount of PAHs was quantified against a calibration curve. For each microcosm experiments, a calibration curve containing the same PAHs used in the experiments was set-up. The excitation and emission wavelengths for each PAH were chosen to obtain the largest peak height accordingly to its spectrum.

Table 4. Program of the fluorescence detector of the HPLC.

Time (minutes)	Event	Wavelength (nm)
0.1	Excitation	275
	Emission	330
16.4	Excitation	250
	Emission	390
20.2	Excitation	285
	Emission	400
21.7	Excitation	270
	Emission	390
29.6	Excitation	290
	Emission	430

2.1.11 Amplification of DNA by Polymerase Chain Reaction (PCR)

The extracted DNA was amplified by Polymerase chain reaction. PCR uses the double stranded DNA to be copied (the DNA template), oligonucleotide primers (short segments of single-stranded DNA, each of which is complementary to a short sequence on one of the strands of the template DNA), nucleotides, and thermo stable polymerase enzyme (*Taq*). These 4 ingredients are mixed and heated, causing the denaturation of the DNA, in other words, the DNA separates in 2 single strands. The mixture is cooled and the primers attach to the complementary sites on the template strands, then the polymerase copy the template strands by adding nucleotides onto the ends of the primers, resulting in 2 molecules of double-stranded DNA. The cycle is repeated for several times, increasing the amount of DNA exponentially. Thirty cycles generate approximately 1 billion copies of the original DNA sequence (Mullis, 1990).

In the present work two sets of primers were used, one for amplification of DNA from isolated bacteria, 16F27 (AGA GTT TGA TCM TGG CTC AG) and 16R1492 (TAC GGY TAC CTT GTT ACG ACT T); and other for amplified DNA from communities, Com1F and Com2-PhR. Both amplify a region of the DNA that code for 16S ribosomal RNA (rRNA), a region of approximately 1,520 nucleotides that is widely used for the analysis of the phylogeny of microorganisms. 16S rRNA are extremely well conserved molecules in overall structure and a significant component of the cellular mass (Olsen *et al*., 1986), where they exhibit 9 small variable regions which allow species differentiation, thus identification. The primers Com1 (forward - 5’CAGCAGCCGCGGTAATAC-3’) bind to the positions 519 to 536 of 16S rRNA and primer Com2-Ph (reverse - 5’-CCGTCAATTCCTTTGAGTTT-3’) bind to the positions 907 to 926. Com2-Ph contained a 5’-terminal phosphate group (Schwieger and Tebbe, 1998).

The PCR reactions were performed in a thermocycler in a total volume of 50µl, as described in supplementary tables 1 to 4. The PCR products obtained from the 16S rRNA reaction were purified with the PCR Clean-up Gel extraction kit NucleoSpin Extract II (Macherey Nagel, Düren, Germany), while PCR products for SSCP were purified with the MiniElute kit (QIAGEN, Hilden, Germany), both following the manufacturer’s instructions.

2.1.12 Community fingerprinting

2.1.12.1 SSCP fingerprint analysis of the bacterial community

Single Stranded Conformation Polymorphism (SSCP) is a technique for genetic profiling of microbial communities based on PCR-amplified signature genes. It is an important technique to evaluate natural variability between microbial communities, i.e., in response to environmental changes. It is a culture-independent method that contributes to both fast differentiation and identification of microorganisms, even for those microorganisms which have not yet been cultured in the laboratory (Schmalenberger *et al*., 2001).

The PCR product from the reaction with Com primers was digested with 2.5µl lambda exonuclease buffer and with 2.5 µl lambda exonuclease for 1 hour at 36°C. This enzyme removes the phosphorylated strand, resulting in a single strand DNA product that was purified with MiniElute kit (QUIAGEN, Hilden, Germany) following the manufacturer's instructions. The amount of DNA was measured in a NanoDrop spectrophotometer and 100 ng of DNA was dried in a vacuum centrifuge. DNA was resuspended in 4 µl water and 4 µl denaturating SSCP loading buffer and subjected to SSCP in a polyacrylamide gel (Schwieger and Tebbe, 1998). The gel consisted of 9 ml MDE^{TM} gel (2-fold concentrated solution Acrylamide, BMA Lonza, Rockland, USA); 3 ml 10xTBE buffer; 18 ml bidistilled water; 120 µl 10% ammonium persulfate (APS) and 12 µl N,N,N',N' tetraethylmethylendiamin (TEMED, Amresco, Solon, Ohio, USA). Before pouring the gel, two glass plates were washed with detergent and water and cleaned with 70% ethanol. One glass plate was then coated with a solution of 3 ml ethanol p.a.; 30 µl of acetic acid and 30 µl of Bind-silane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The other glass plate was treated with 500 µl of Repel-silane ES (Amersham Biosciences, Uppsala, Sweden). Both plates were cleaned with 70% ethanol after 10 minutes. Spacer (0.4 mm) were placed between the plates and secured with clamps. The gel was poured between two glass plates and allowed to polymerise for 2.5 hours.

The SSCP chamber was filled with TBE buffer and the apparatus was connected to a cooling water system adjusted to 20°C. The 8 µl sample was loaded into the gel pockets and it ran at 400 V for 16 h. In each gel 0.25 µg (1 µl) of DNA Molecular Weight Marker III 0.12 – 21.2 kbp (Roche) mixed with denaturating SSCP

loading buffer (3µl of) was also loaded into three gel pockets. After the run, the gels were silver stained according to Bassam *et al*. (1991) (Supplementary Table 5).

The bands obtained in the SSCP gel were excised and DNA was extracted with 50 µl of SSCP elution buffer (SSCP EB) at 96°C for 15 minutes. The extracted DNA was amplified by Com PCR and purified for the sequence reaction.

2.1.12.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP, a high-throughput quantitative approach was performed on some samples in microcosm experiments 2 and 6 in order to confirm the semi-quantitative approach of SSCP to investigate patterns within the microcosm experiments.

For PCR amplification, the 5' end fluorescent-labelled: 27F-FAM and 1492R-VIC primers targeting the 16S rRNA gene (Applied Biosystems, Carlsbad, CA, USA) were applied (as already described above). PCR reactions (50 µl) were performed at an annealing temperature of 55°C using 2.5 ng DNA as template and purified using the Macherey-Nagel 96-well plate purification kit (Macherey-Nagel, Düren, Germany). Exo-Klenow fragment treatment was performed, where 10 U of exo-Klenow was incubated with 80 ng of amplicons in a volume of 40 µl (1 h, 20°C) followed by inactivation (20 min, 75°C). Digestion was then performed with 2.5 U of *AluI* (3 h, 37°C) followed by heat inactivation (20 min, 65°C) and purification through DyeEx gel-filters (Qiagen) with re-elution into 40 µl of water. Triplicate 10 µl aliquots containing 20 ng of digested fluorescent-labelled DNA fragments for each sample were dried, resuspended in 9.75 µl HiDi formamide (Applied Biosystems) and 0.25 µl of GeneScan 500 LIZ size standard (Applied Biosystems), and denatured (3 min, 95°C). DNA fragments were separated on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems). Any fragment of 35 nucleotides or less was excluded. T-align (http://inismor.ucd.ie/~talign) was used to normalize total peak area across replicates and align peaks across samples using a +/-0.5 bp cut-off (Smith *et al.,* 2005).

2.1.13 Sequencing reaction

To proceed with the phylogenetic analysis, the PCR products (16S rRNA or Com PCR) were sequenced by the Sanger method (chain-termination). In this method a reaction is made similar to a normal PCR with the addition of dideoxynucleosidetriphosphates (ddATP, ddGTP, ddCTP, or ddTTP) fluorescent labelled, but in lower concentration than the deoxynucleotides. They are chain-termating nucleotides because whenever they are added to the new synthesized DNA strand the polymerase cannot add any other nucleotide due to the lack of 3'-OH group required for the formation of a phosphodiester bond between two nucleotides during DNA strand elongation, so they terminate the DNA strand extension, resulting in various DNA fragments of different length. The lower concentration of the dideoxynucleotides allows strand elongation sufficient for sequence analysis. The parameters for the sequencing reaction are described in supplementary tables 6 and 7.

Isolates were sequenced using 16S rRNA primers: 16R1087 (5'-ACT GGC GGA CGG GTG AGT AA-3'), 16F945 (5'-TAC GGY TAC CTT GTT ACG ACT T-3'), 16R518 (AGA GTT TGA TCM TGG CTC AG-3'), 16F357 (5'-CCG CTT GTG CGG GCC CCC GTC-3') and SSCP bands with primers Com1F and Com2-PhR. Each primer was employed in a separate reaction.

The products of the sequencing reactions were purified with DyeEx 2.0 Spin Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified sequencing reaction products were dried in a vacuum centrifuge and sequenced by employing a 3130*xl* Genetic Analyzer (Applied Biosystems, Darmstadt).

The gene sequences were annotated using Sequencher^{TM} 4.8 (Gene Codes Corporation, Ann Arbor, USA). Phylogenetic and molecular evolutionary analyses were conducted using BioEdit 7.0.5.3 (Hall, 1999). Sequence Alignment Editor and phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura *et al*., 2007).

2.1.14 Microscopy

2.1.14.1 Confocal Laser scanning microscopy (CLSM)

Confocal Laser scanning microscopy (CLSM) was performed using the model TCS SP (Leica, Heidelberg, Germany) attached to an upright microscope. The instrument was controlled by Leica Confocal software, version 2.5, Build 1347d. The system was equipped with three visible lasers: an Ar laser (458, 476, 488, and 514 nm), a laser diode (561 nm), and a He-Ne laser (633 nm). The spectrophotometer feature allowed flexible and optimal adjustment of sliders on the detector side.

The biofilm samples were stained for 15 minutes with Syto 60 or *Aleuria aurantia* lectin at room temperature, the sample was carefully rinsed twice and observed by CLSM microscopy. The following settings were used for excitation of Syto green, 663 nm and *Aleuria aurantia* lectin 660 nm. All samples were examined immediately after staining using CLSM. Biofilm samples were observed with 10x 0.3-numerical aperture (NA), 20x0.5-NA, and 63x0.9-NA water-immersible lenses.

2.1.14.2 Field emission scanning electron microscopy

Samples were fixed in 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose, pH 6.9) overnight at 4°C, washed several times with cacodylate buffer and subsequently washed with TE-buffer (20 mM TRIS, 1 mM EDTA, pH 6,9) before dehydrating in a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 15 minutes for each step. Samples in the 100% acetone step were allowed to reach room temperature before another change in 100% acetone. Samples were then subjected to critical-point drying with liquid CO_2 (CPD 30, Bal-Tec, Liechtenstein). Dried samples were then mounted onto conductive carbon adhesive tabs on a aluminium stub and sputter coated with a thin gold film (SCD 500, Bal-Tec, Liechtenstein) before examination in a field emission scanning electron microscope Zeiss DSM 982 Gemini using the Everhart Thornley SE-detector and the inlens SE-detector in a 50:50 ratio at an acceleration voltage of 5 kV and at calibrated magnifications. Images were recorded onto MO-disk and contrast and brightness were adjusted using Adobe Photoshop CS3.

2.1.15 Preparation of Fatty Acid Methyl Esters (FAMES)

Slides with biofilms were cut and placed inside an extraction flask where a solution of methanol and sodium hydroxide (MeOH:NaOH 15%; 1:1 v/v) was added and agitated for 5 minutes to remove the cells. The solution was transferred to a 4 ml glass vial and securely sealed with a teflon lined cap. The suspension was heated for 1h at 100°C in a heating block. The solution was cooled to room temperature and 1.8 ml of a solution of hydrochloric acid and methanol (MeOH:HCl 37%; 10:2 v/v) was added to methylate the saponificated fatty acids followed by another heating step at 80°C for 10 minutes. The reaction was quickly stored on ice until cooled. For the extraction of the fatty acid methyl esthers (FAMEs) from the aqueous phase 0.9 ml of a solution hexan/ter-butyl methyl ether solution (1:1 v/v) was added. After vigorous vortexing for 30 seconds the upper organic phase was transferred to a new 4 ml-vial. This extraction step was performed three times. To prevent any contamination from the organic phase during gas chromatography analysis 3 ml of basic solution (0.5 M sodium hydroxide) was added. After vortexing again for 30 seconds the organic phase was transferred to a 2 ml crimp top vial. The solvent was evaporated by a gentle stream of nitrogen. For the gas chromatographic analysis FAMEs were resuspended in octane.

In an attempt to remove the PAH from the solution, the protocol was modified at the first step of the extraction where MeOH:NaOH is added, by adding first 2 ml of hexane or dichloromethane to extract the PAHs and 1.5 ml of water to separate the cells from the PAHs. The organic phase was removed, the aqueous phase was centrifuged to sediment the cells and the supernatant was removed. Then MeOH:NaOH was added and the FAMES protocol was followed. Another modification to the protocol was done by adding hexane or dichloromethane directly to the MeOH:NaOH solution to extract the PAHs, and removing the solvents after 30 seconds.

The FAMES were analyzed in an Agilent 6890N gas chromatograph equipped with an 5%-PhenylMethylpolysiloxan Optima 5 capillary column; 50 m length; 0.32 mm inner diameter; 0.25 µm film thickness and a Flame Ionization Detector (FID). Hydrogen served as the carrier gas. The injector temperature was set at to 250°C and detector temperature was 300°C. The oven program for the FAMEs analysis

was 100°C for 2 minutes, subsequently increasing the temperature to 290°C at 4°C/ min and isothermal period of 10 minutes.

2.1.16 Statistical analysis

Non-parametric multivariate statistical analysis was performed using PRIMER (v.6.1.6, PRIMER-E, Plymouth Marine Laboratory, UK) (Clarke, 1993; Clarke and Warwick, 2001). All multivariate routines were computed on semi-quantified SSCP data. From the multivariate data matrix, a sample-similarity matrix was generated using the Bray-Curtis similarity coefficient by comparing the abundances of each of the SSCP bands in regards to every pairwise combination of all samples. Community structures were explored by both ordination using nonmetric multidimensional scaling (nMDS) (50 random restarts) and cluster analysis using the group-average algorithm. For nMDS ordination, a stress value below 0.2 corresponds to a useful ordination (Clarke and Warwick, 2001). Data are presented in such a way that samples with a similar community composition are closer in spatial proximity on the plot, while samples that are more spatially separated on the plot have less species groups in common. The relative distance between samples on the plot is thus not arbitrary (Clarke, 1993). As the nMDS plot is, however, arbitrarily oriented, axes are usually not presented (Clarke, 1993).

For analyzing each of the single variables at a time, non-parametric univariate statistical analysis was performed using GraphPad Prism (v.4.0, GraphPad Software, Inc.). Analysis of Variance (two-way ANOVA) was performed on each of the individual univariate variables (PAHs). Treatment and time are treated as fixed factors. Groups of samples are considered significantly different if the p-value falls <0.05 and highly significantly different if the p-value falls <0.01.

2.2 Materials

Chemicals

The solvents used in the present work were purchased from J. T. Baker (Deventer, Netherlands) and Merck (Darmstadt, Germany) in p.a. quality.

Other chemicals were supplied from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), and Roth (Karlsruhe, Germany).

PAHs were purchased from LGC Standards, Wesel, Germany.

Lambda exonuclease and lambda exonuclease buffer were supplied from New England Biolabs, Schwalbach, Germany; the primers were from Invitrogen, Karlsruhe, Germany, and the reagents for PCR (*Taq*, buffer, dNTP, $MgCl_2$) were supplied by QUIAGEN.

Compositions of buffers, media and solutions as well as equipments utilized in this work are described in the supplementary material sections 7.4 and 7.5.

3 Results

The degradation of 16 different PAHs by mixed communities was assessed by conducting a range of microcosm experiments, where the PAHs were extracted from the slides and analyzed by HPLC. Similarities between community structures over time was assessed by SSCP (and in two cases T-RFLP) followed by statistical ordination and clustering techniques. As naphthalene amount was below the limit of detection in the majority of samples, due to losses because of evaporation, this PAH was not included in the degradation analysis.

3.1 Microcosm experiment 1: Waldau Oderteich

3.1.1 Microcosm experiment 1 – Part 1: Waldau, Oderteich

The first microcosm experiment was performed with samples from Waldau, site 3 (W3) and site 7 (W7); and Oderteich site 1 (Od1) and 2 (Od2). Samples were taken at 1, 7, 14, 21, 28, 35, 42, 90 and 180 days in order to measure PAH degradation and to analyse the communities formed on the crystals.

3.1.1.1 Degradation analysis

This microcosm experiment showed that of the four sediment samples used (W3, W7, Od1 and Od2), the community of W3 was the only community able to degrade most of the PAHs tested, even the HMW PAHs. Figure 13 depicts the LMW PAH degradation of microcosms W3 and Od1, except for acenaphtylene which has a similar pattern to that of acenaphtene and fluorene. Figures 14 and 15 show the degradation of HMW PAHs, except benzo(g,h,i)perylene and dibenz(a,h)anthracene that were not significantly degraded. The communities of W7 and Od2 did not degrade any of the PAHs (data not shown). In all microcosms, abiotic processes such as solubility and detachment of the crystal from the Permanox® slide are considered the cause of the decline amount of PAH in the blank samples during the

first days of incubation. This process occurs in all microcosms and continues during the whole experiment but at a lesser rate. The blank used in all experiments was soil autoclaved three times. It cannot be excluded that some fungal spores may resist the sterilisation and become viable during the microcosm incubation time.

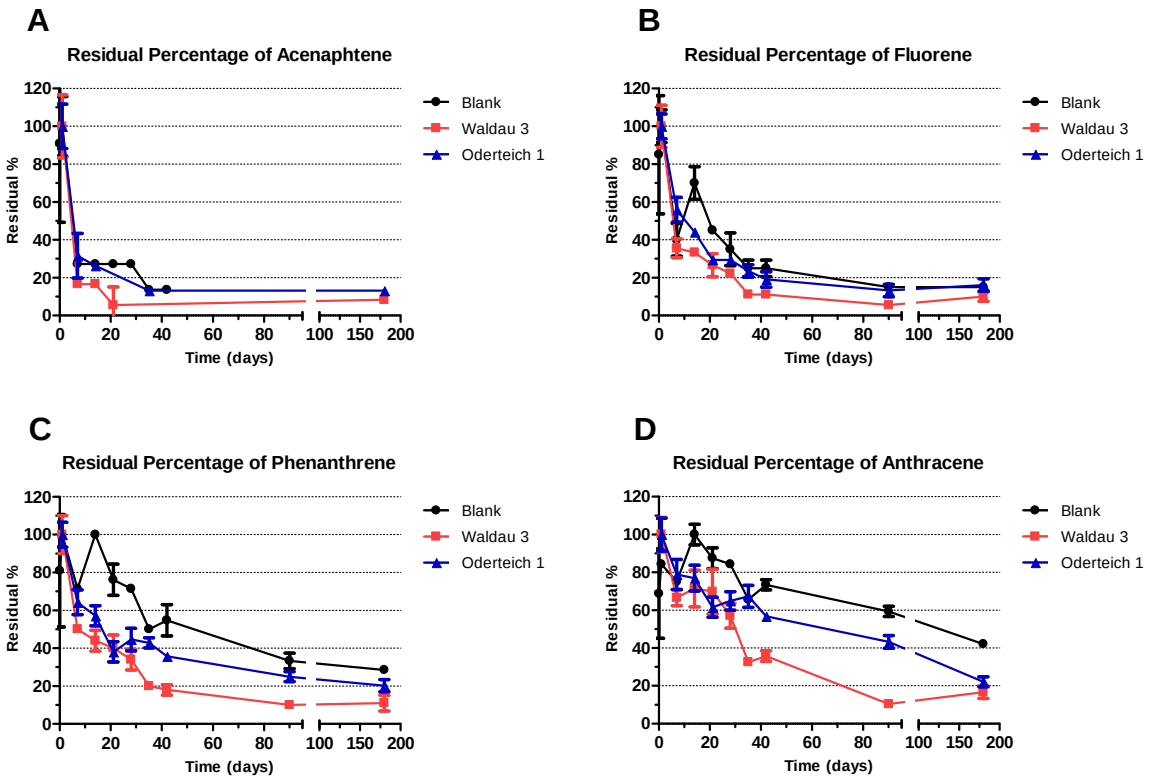


Figure 13. Degradation curve of LMW PAHs in microcosm W3 and Od1. (A) acenaphthene, (B) fluorene, (C) phenanthrene, and (D) anthracene.

Within 14 days, the W3 community has degraded the LMW PAHs at some extent but this became more significant after 35 days of incubation (ANOVA $P<0.05$, see supplementary material section 7.6.1 tables S8-S10). It seems that after 42 days the PAH reached its maximum degradation rate in all microcosms, except for anthracene. Anthracene is the LMW that is more difficult to degrade, but still reached its maximum degradation at 90 days, when its amount stabilized.

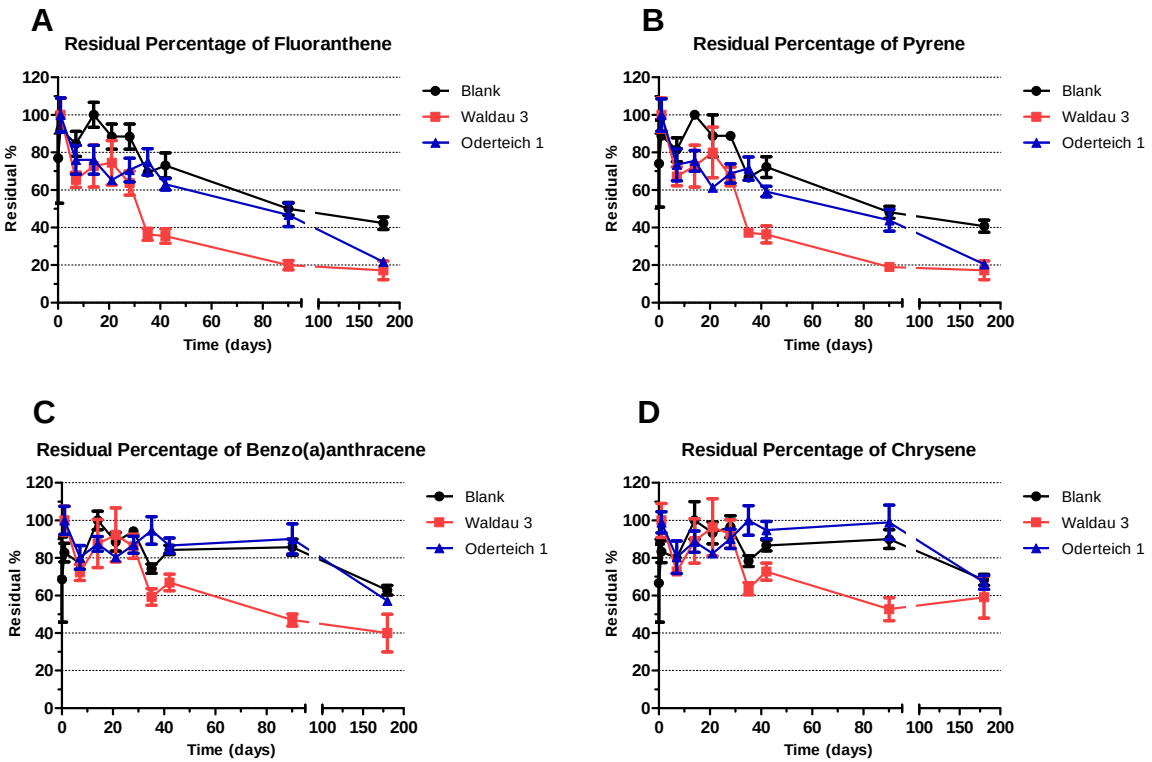


Figure 14. Degradation curve of HMW PAHs in microcosms W3 and Od1. (A) fluoranthene, (B) pyrene, (C) benz(a)anthracene, and (D) chrysene.

HMW PAH degradation was also significant within 35 days (ANOVA $P<0.05$, see supplementary material section 7.6.1 tables S11-S20), and after 90 days, the degradation rate slowed down, but continued. The amount of indeno(1,2,3-*c,d*)perylene was not detectable at 180 days of incubation (Figure 15).

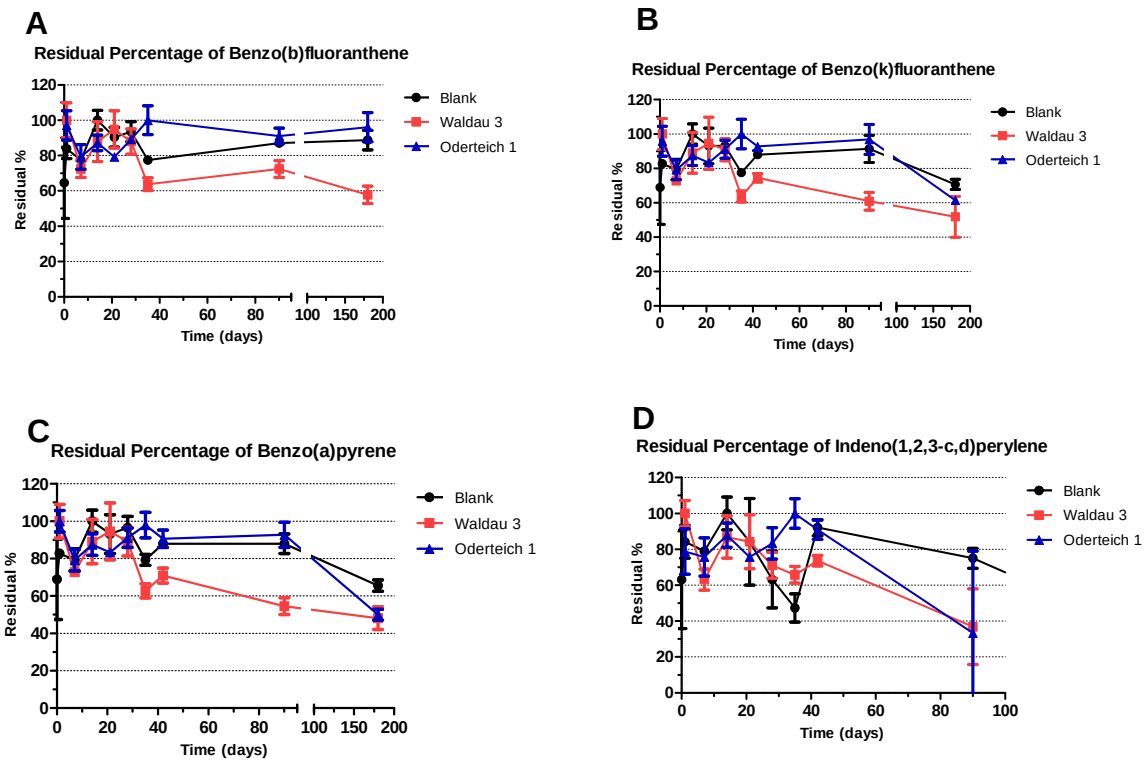


Figure 15. Degradation curve of complex HMW PAHs in microcosms W3 and Od1. (A) benzo(b)fluoranthene, (B) benzo(k)fluoranthene, (C) benzo(a)pyrene and (D) indeno(1,2,3-*c,d*)perylene.

3.1.1.2 Community structure analysis

All the communities of microcosm experiment 1 (W3, Od1, W7 and Od2) presented several community members. Inspecting the SSCP gel (Figure 16) it was possible to conclude that the communities from Oderteich (Od1 and Od2) shared a lot of SSCP bands, although Od1 presented higher diversity. However, the communities of W3 and W7 presented very different SSCP bands, thus differences in their community structure may explain the different degradation rates of PAHs by these samples. Based on these results, where the best degradation was promoted by the W3 community, this community was studied in more details.

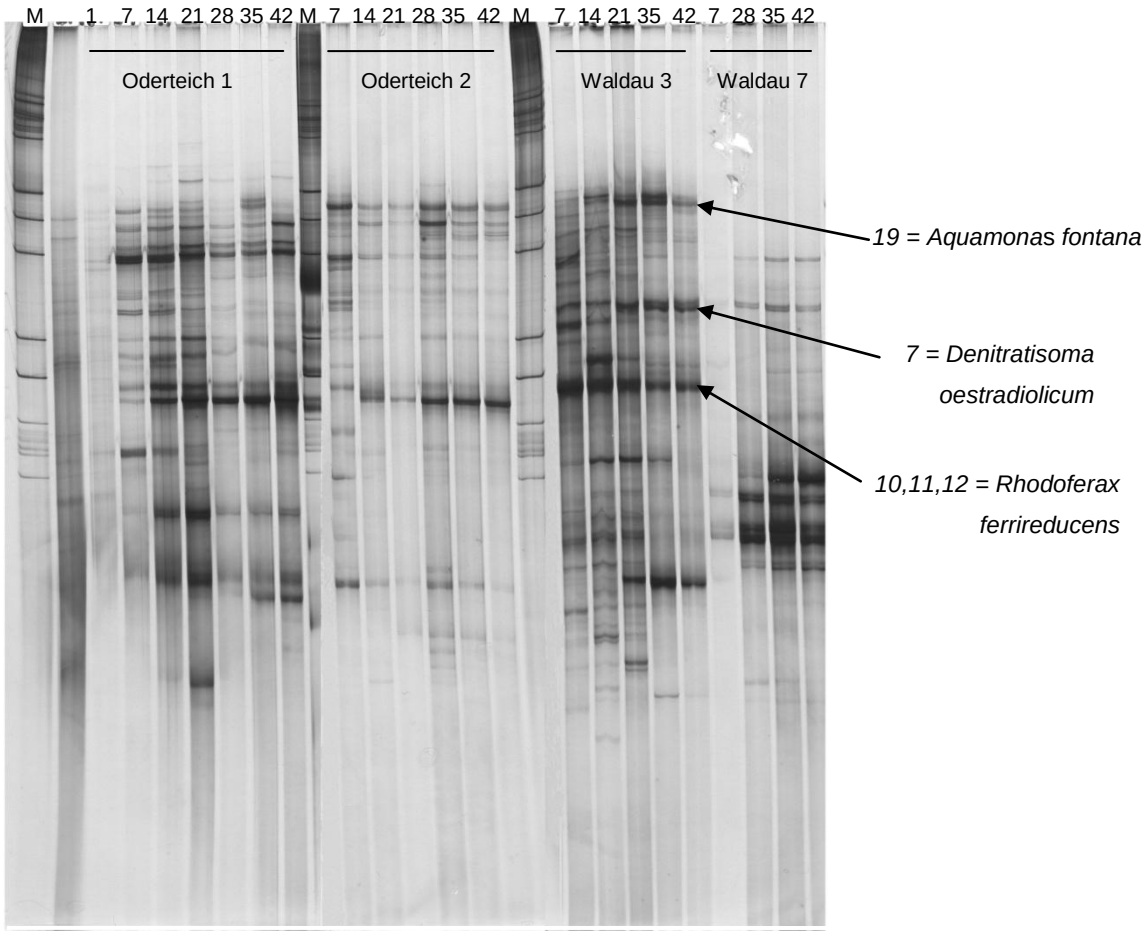


Figure 16. SSCP gel of microcosm experiment 1. Numbers on top of the gel correspond to sampling time in days and M correspond to the lane of the marker. Numbers besides the genus correspond to the respective bands.

Bands from the SSCP gel corresponding to microcosm W3 (Figure 16) were excised, the DNA was extracted and sequenced. The members of this community belong to the class *Proteobacteria*, except one for which the closest related genus was *Flectobacillus*. Taxa belonging to the genera *Rhodoferax ferrireducens* (98.9% similarity), *Aquamonas fontana* (98.4% similarity) and *Denitratisoma oestradiolicum* (94.4% similarity) were found to be dominant and, interestingly after 42 days of incubation there were almost no other genera present in the community besides these. The following phylogenetic tree (Figure 17) presents the closest related genus from each sequence obtained.

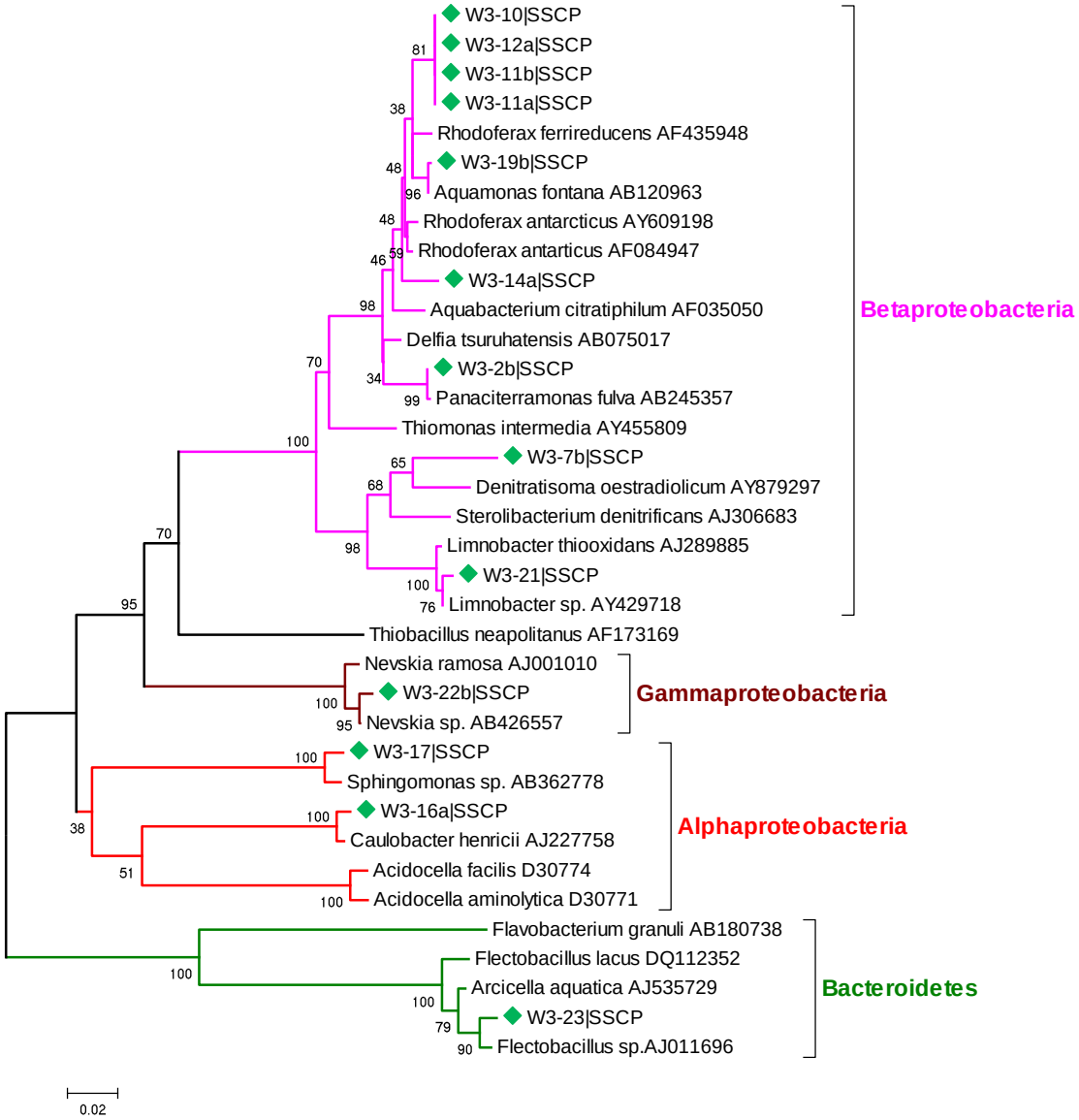


Figure 17. Phylogenetic positions of sequences from the SSCP bands from microcosms W3 (green diamonds) based on neighbour joining clustering after multiple alignment (360 bp) of the 16S rRNA gene. Branch colour denotes class-level.

Confocal microscopy showed that the bacteria colonised the PAH crystals (Figure 18A) and formed biofilm on them, as is possible to see in Figure 17B, where the extrapolymeric material is dyed with *Aleuria aurantia* lectin. Although the lectin has broad specificity, it binds preferentially to fucose molecules and it does not enter the cells.

By the field emission scanning electron microscopy it is clear that a biofilm has formed due to the polymeric material present (Figure 19A, arrows) and various species are present on the biofilm (Figure 19B and C). Unfortunately, the PAH

crystals cannot be seen on the micrographs because they were solubilised and lost during the drying process of the samples once different concentrations of acetone were applied.

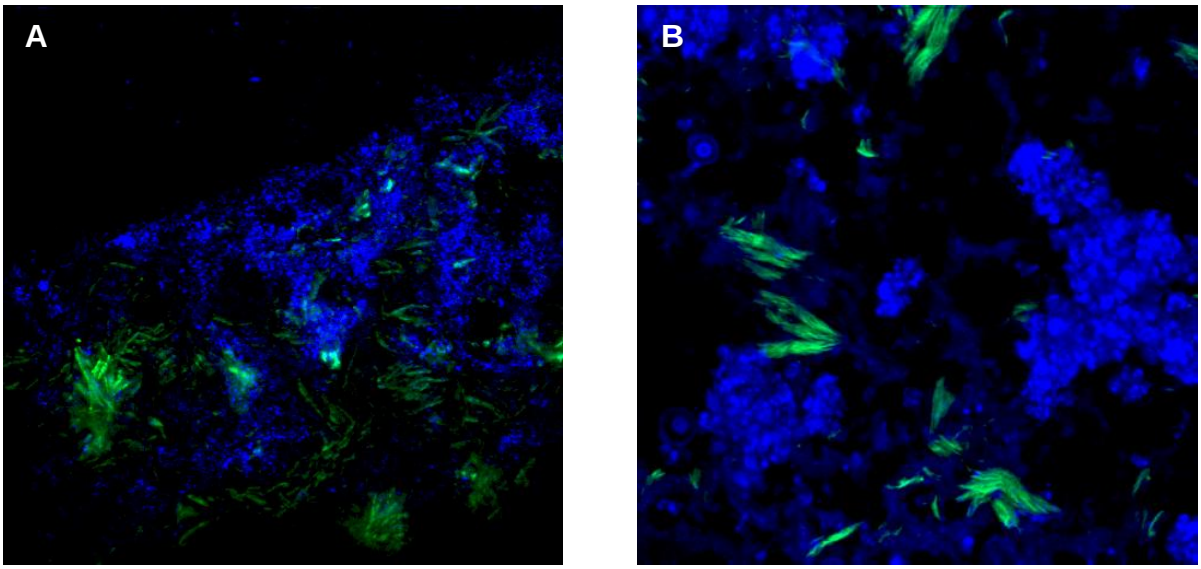


Figure 18. Confocal microscopy performed with communities from W3. Green colour is the autofluorescence of PAH crystals. (A) Blue is DNA dyed with Syto 60. (B) Blue is extrapolymeric material dyed with *Aleuria aurantia* lectin (AAL_A633). Photo: Dr. Thomas Neu

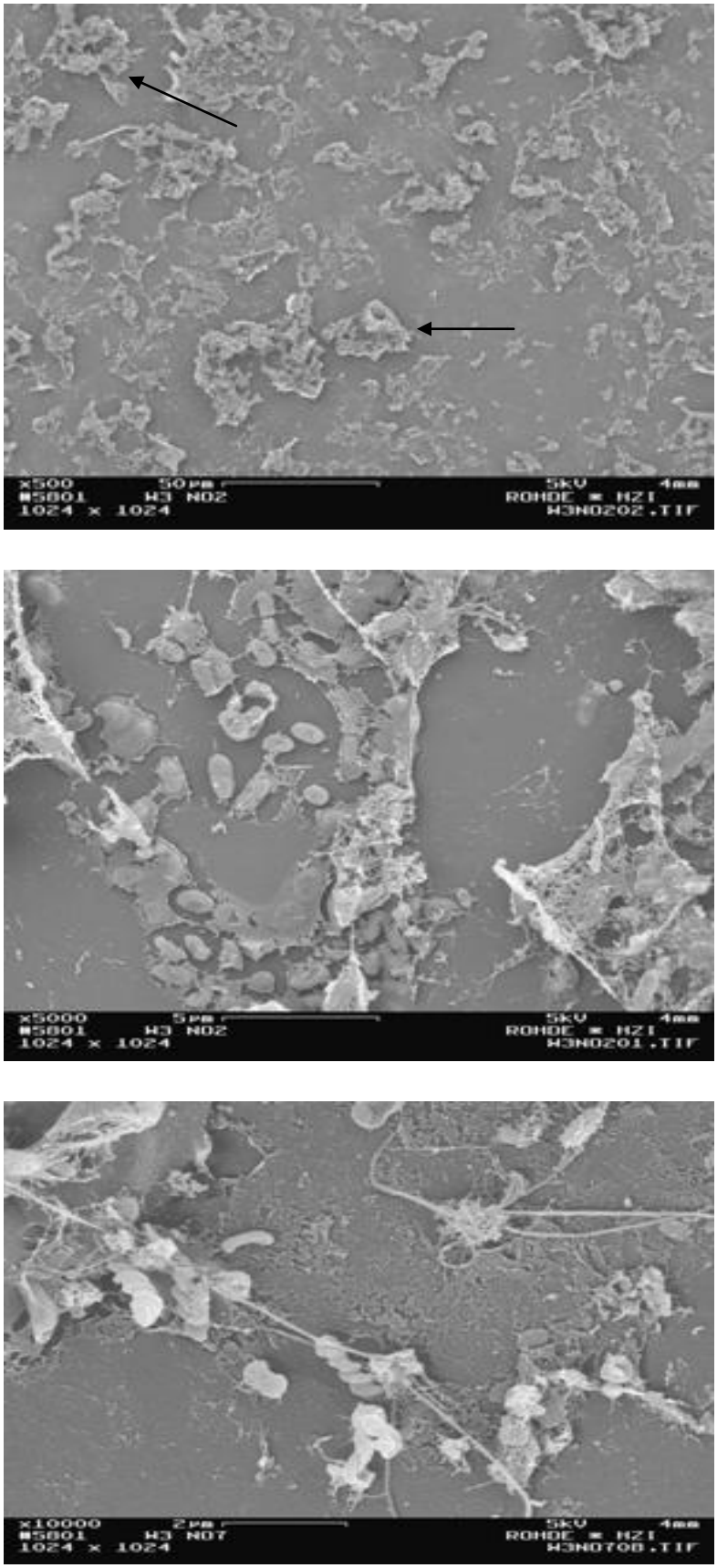


Figure 19. Field emission scanning electron microscopy of communities from W3. Photo: Dr. Manfred Rohde.

3.1.2 Microcosm experiment 1 – Part 2: W3 Single crystals

After working with a mixture of PAHs, it was decided to use single crystals of each PAH as substrate in microcosm W3 after being enriched for 5 months to observe whether the communities were dependent upon the substrate or would they remain the same for every PAH. After 60 days of incubation the slides with single PAH crystals were taken and the community analysed.

The analysis of the PAH single crystals showed that the community was dependent upon the substrate, but complex HMW PAHs kept the same community structure, as is shown in Figure 20.

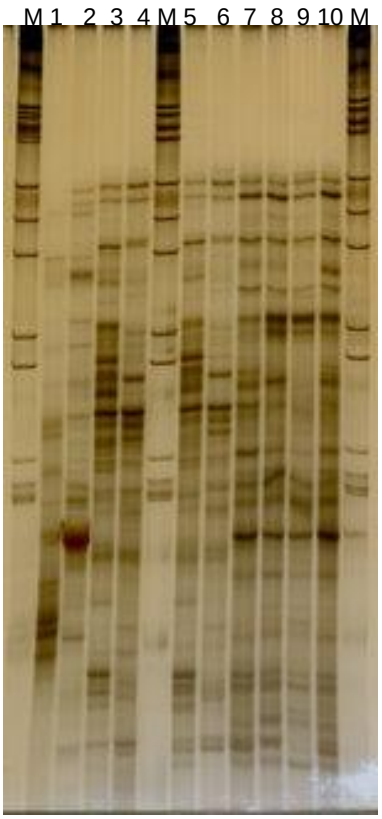


Figure 20. SSCP gel of DNA extracted from the PAH single crystals. Lane M contains the marker while, lane 1 the community formed on naphtalene crystals, lane 2 fluoranthene, lane 3 and 5 anthracene (lane 3, DNA extracted by heating at 96°C with TBE buffer and, lane 5 DNA extracted with SpinKit for Soil Samples), lane 4 and 6 pyrene (lane 4, DNA extracted by heating at 96°C with TBE buffer and lane 5, DNA extracted with SpinKit for Soil Samples), lane 7 benzo(b)fluoranthene, lane 8 benzo(a)pyrene, lane 9 benz(a)anthracene, and lane 10 Indeno(1,2,3-*cd*)perylene.

After SSCP analysis, a presence/absence data matrix of each SSCP band was constructed. It is very interesting to note that after 60 days the bacterial communities formed on anthracene and pyrene substrate, shared just 55% of their members, while the communities after incubation with benzo(b)fluoranthene, benzo(a)pyrene, benz(a)anthracene and indeno(1,2,3-*c,d)*perylene share 88% similarity. Unfortunately, the DNA extracted from the other crystals was not in sufficient amount to form defined bands on the SSCP gel, therefore they weren't included in the analysis. However, with the light bands generated by the mentioned compounds it is already possible to affirm that the SSCP profile of LMW was different from each other, but the communities from HMW PAHs share high similarity between the different compounds, except for pyrene. The amplification of the DNA extracted from the SSCP bands of this gel did not provide sufficient amount to proceed with sequence analysis, consequently it was not possible to identify the members of the communities.

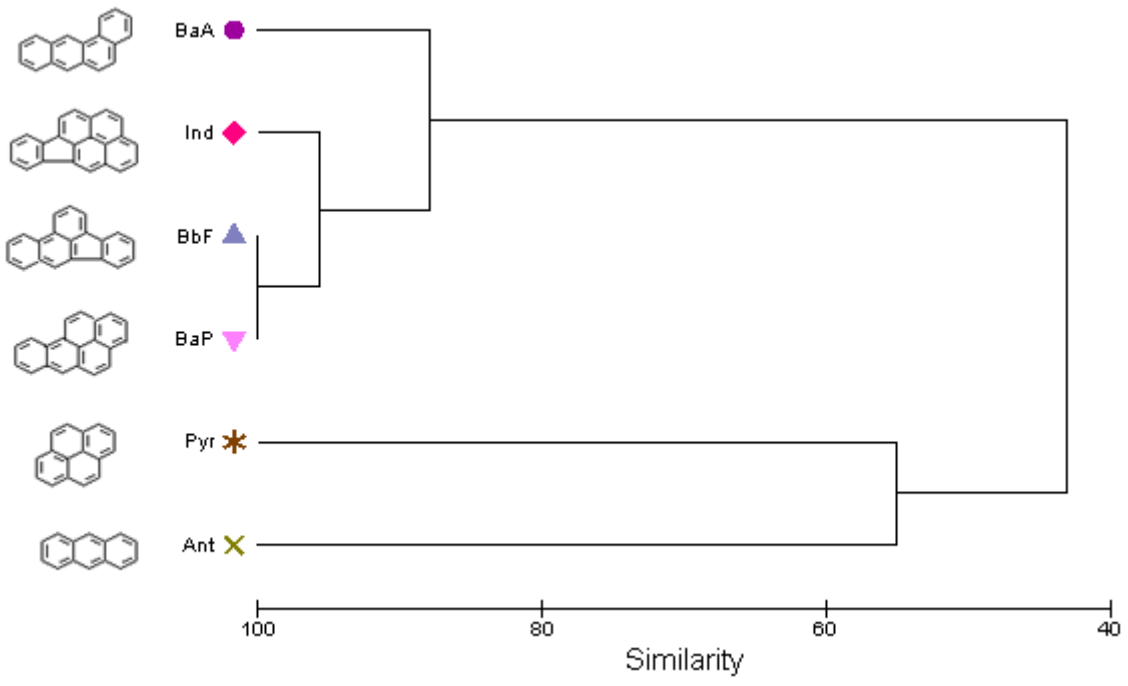


Figure 21. UPGMA cluster analysis of PAH single crystals performed using DNA from the bacterial community after SSCP separation of the 16S rRNA gene and then the Bray-Curtis similarity algorithm. BaA, benz(a)anthracene; Ind, Indeno(1,2,3-*cd*)pyrene; BbF, benzo(b)fluoranthene; BaP, benzo(a)pyrene; Pyr, pyrene; and Ant, anthracene.

It is important to point out that all slides were placed inside the same microcosm (see Figure 10, section 2.1.3.1) and at the same time, therefore the results are not biased due to different conditions. Thus, different communities were selected based on the compound that they were incubated with, and HMW PAHs with similar structures tend to select for similar communities.

3.2 Microcosm 2: Abtsdorf

The analyses of the first experiment (Waldau-Oderteich) showed that it was possible to detect degradation of PAHs within 28 to 42 days, thus for the microcosm constructed with samples from Abtsdorf it was decided to run the experiment for up to 60 days as to screen for the best site at this location.

In this experiment, the smaller PAHs acenaphtene, fluorene, and acenaphtylene (data not shown) were not degraded significantly compared to the blank, although phenanthrene and anthracene presented significantly reduced amount at 60 days of incubation in the slides colonized by the microorganisms from site Abtsdorf B (Figure 22) (ANOVA $p<0.05$, see supplementary material section 7.6.2 tables S21-S24).

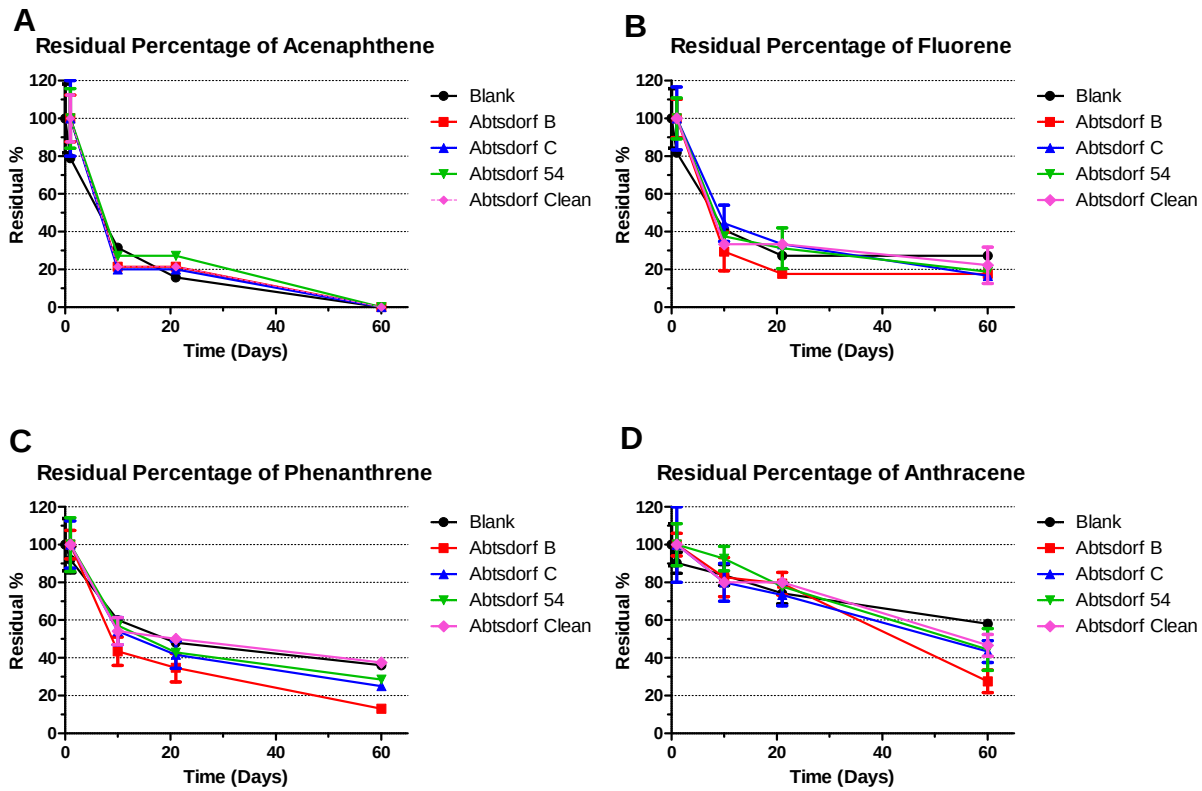


Figure 22. Degradation curve of LMW PAHs in microcosm Abtsdorf. (A) acenaphthene, (B) fluorene (C) phenanthrene and (D) anthracene.

On HMW PAHs, degradation was also observed in 60 days of incubation, and fluoranthene, pyrene, benz(a)anthracene, and chrysene were the compounds most degraded within this time (ANOVA $p<0.05$, see supplementary material section 7.6.2 tables S25-S34), again in the microcosm inoculated with Abtsdorf B, but also with Abtsdorf C (Figure 23). Data related to benzo(g,h,i)perylene and dibenz(a,h) anthracene were inconclusive, therefore these graphs are not shown.

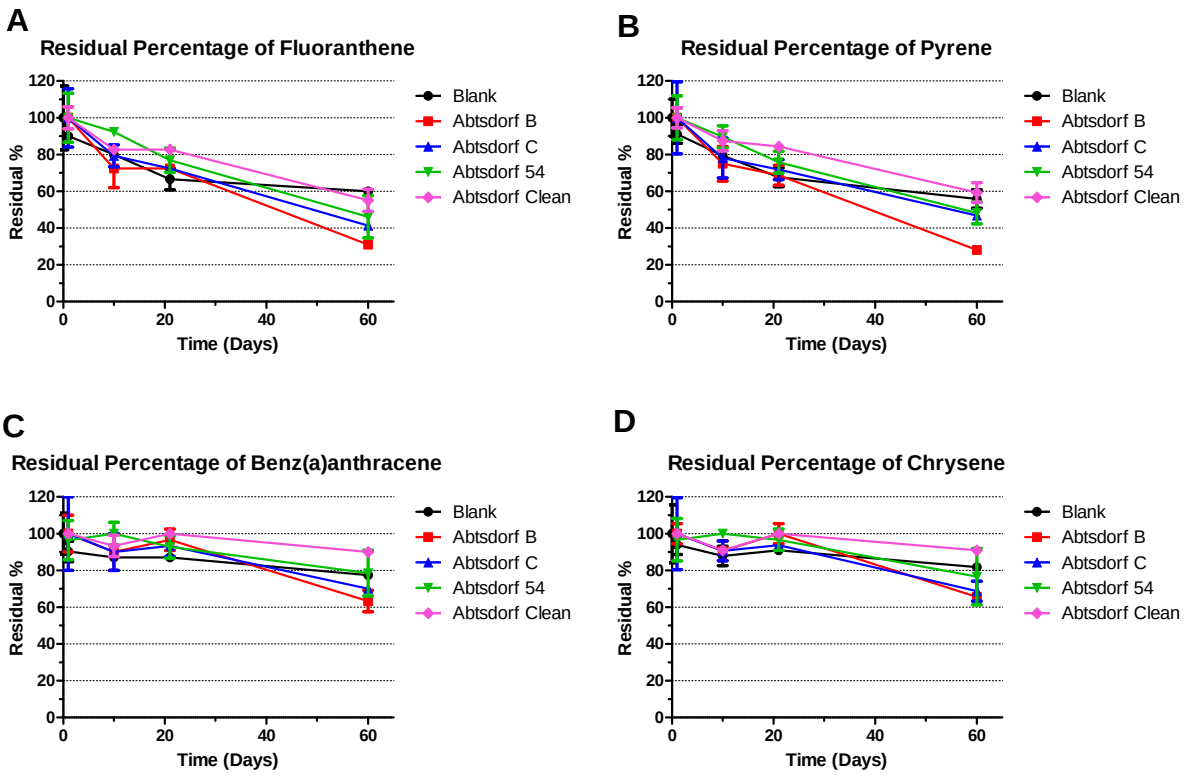


Figure 23. Degradation curve of HMW PAHs in microcosm Abtsdorf. (A) fluoranthene, (B) pyrene, (C) benz(a)anthracene and (D) chrysene.

The T-RFLP analysis of the soil from Abtsdorf showed that the community from site B is only 50% similar to the others, whereas soil C and 54 present about 75% similarity, which might explain the similar degradation profile of these two samples (Figure 24). The site called Abtsdorf Clean was the most different from the others, as well as in the degradation profile.

Based on the observations of degradation and on the community differences, bacteria strains were isolated from the biofilm formed on the PAH crystals in the microcosms inoculated with soil from Abtsdorf B and Abtsdorf C.

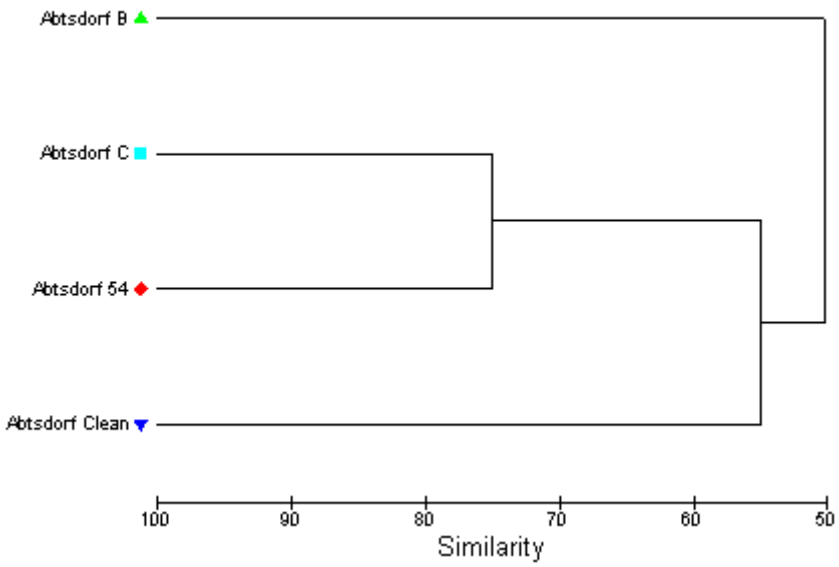


Figure 24. UPGMA cluster analysis of soil Abtsdorf B, C, 54 and Clean performed using T-RFLP data and the Bray-Curtis similarity algorithm..

Isolates from Abtsdorf presented low diversity. Only four genera were identified: *Burkholderia*, *Collimonas*, *Pseudomonas* and *Bacillus*. Although the isolates from both sites were similar, the genus *Bacillus* was just isolated from Abtsdorf B and not from site C. Figure 25 gives an overview of the isolates and their respective closest neighbours.

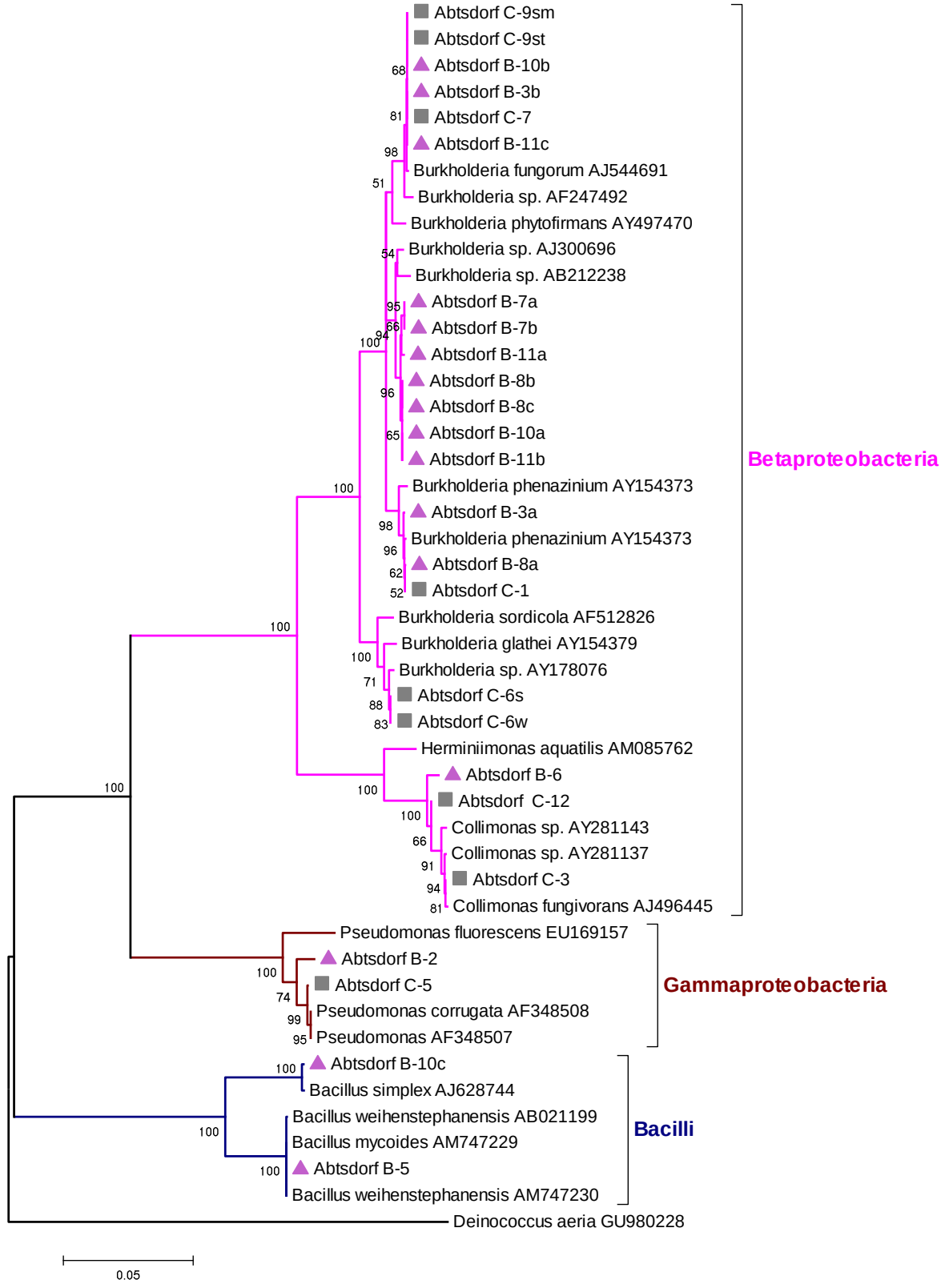


Figure 25. Phylogenetic positions of isolates from Abtsdorf B (pink triangles) and Abtsdorf C (gray squares) based on neighbour joining clustering after multiple alignment (1400 bp) of the 16S rRNA gene sequences. Branch colour denotes class-level. Deinococcus aeria was used as outgroup.

3.3 Microcosm 3: Microcosm W3

The present microcosm experiment was inoculated with either bacteria isolated directly from the sediment W3 (Sed.), with bacteria isolated from the biofilm formed on PAH crystals in the microcosm W3 (Biofilm) or with all these bacteria mixed together (Mix).

3.3.1 Isolates used in Microcosm W3

The bacteria isolate from the sediment W3 and the biofilm were very distinct. Isolates from sediment belong to six genera: *Aeromonas, Arthrobacter, Paenibacillus, Pseudomonas, Streptomyces* and *Yersinia*, where *Pseudomonas* was the most abundant and it was isolated either on plates containing phenanthrene or pyrene crystals. Also from the biofilm six genera were isolated: *Bacillus, Hydrogenophaga, Mycobacterium, Pseudomonas, Sphingopyxis* and *Streptomyces*, but in this case, the most abundant genus was *Bacillus* (Table 5).

The phylogenetic tree constructed with the sequences of all isolates from W3 shows that despite some strains belonged to the same genus, the majority of them are not identical isolates. That was just true for the *Bacillus* sp. isolates W3 B15 and W3 B16 from the biofilm; and isolates from sediment: W3 Phe3, W3 Phe4, W3 Pyr4 and W3 Pyr6, all belonging to the genus *Pseudomonas* (Figure 26).

Table 5. Isolates from Waldau 3

Source	Isolate	Genus
Waldau 3 sediment	W3 Phe1	*Pseudomonas* sp.
Waldau 3 sediment	W3 Phe2	*Pseudomonas* sp.
Waldau 3 sediment	W3 Phe3	*Pseudomonas* sp.
Waldau 3 sediment	W3 Phe4	*Pseudomonas* sp.
Waldau 3 sediment	W3 Ant1	*Yersinia* sp.
Waldau 3 sediment	W3 Pyr1	*Aeromonas* sp.
Waldau 3 sediment	W3 Pyr2	*Streptomyces* sp.
Waldau 3 sediment	W3 Pyr3	*Paenibacillus* sp.
Waldau 3 sediment	W3 Pyr4	*Pseudomonas* sp.
Waldau 3 sediment	W3 Pyr5	*Arthrobacter* sp.
Waldau 3 sediment	W3 Pyr6	*Pseudomonas* sp.
Waldau 3 biofilm	W3 B1	*Bacillus* sp.
Waldau 3 biofilm	W3 B2	*Bacillus* sp.
Waldau 3 biofilm	W3 B3	*Hydrogenophaga* sp.
Waldau 3 biofilm	W3 B4	*Bacillus* sp.
Waldau 3 biofilm	W3 B5	*Pseudomonas* sp.
Waldau 3 biofilm	W3 B6	*Bacillus* sp.
Waldau 3 biofilm	W3 B7	*Bacillus* sp.
Waldau 3 biofilm	W3 B8	*Pseudomonas* sp.
Waldau 3 biofilm	W3 B9	*Sphingopyxix* sp.
Waldau 3 biofilm	W3 B10	*Streptomyces* sp.
Waldau 3 biofilm	W3 B11	*Bacillus* sp.
Waldau 3 biofilm	W3 B12	*Mycobacterium* sp.
Waldau 3 biofilm	W3 B13	*Hydrogenophaga* sp.
Waldau 3 biofilm	W3 B14	*Mycobacterium* sp.
Waldau 3 biofilm	W3 B15	*Bacillus* sp.
Waldau 3 biofilm	W3 B17	*Mycobacterium* sp.

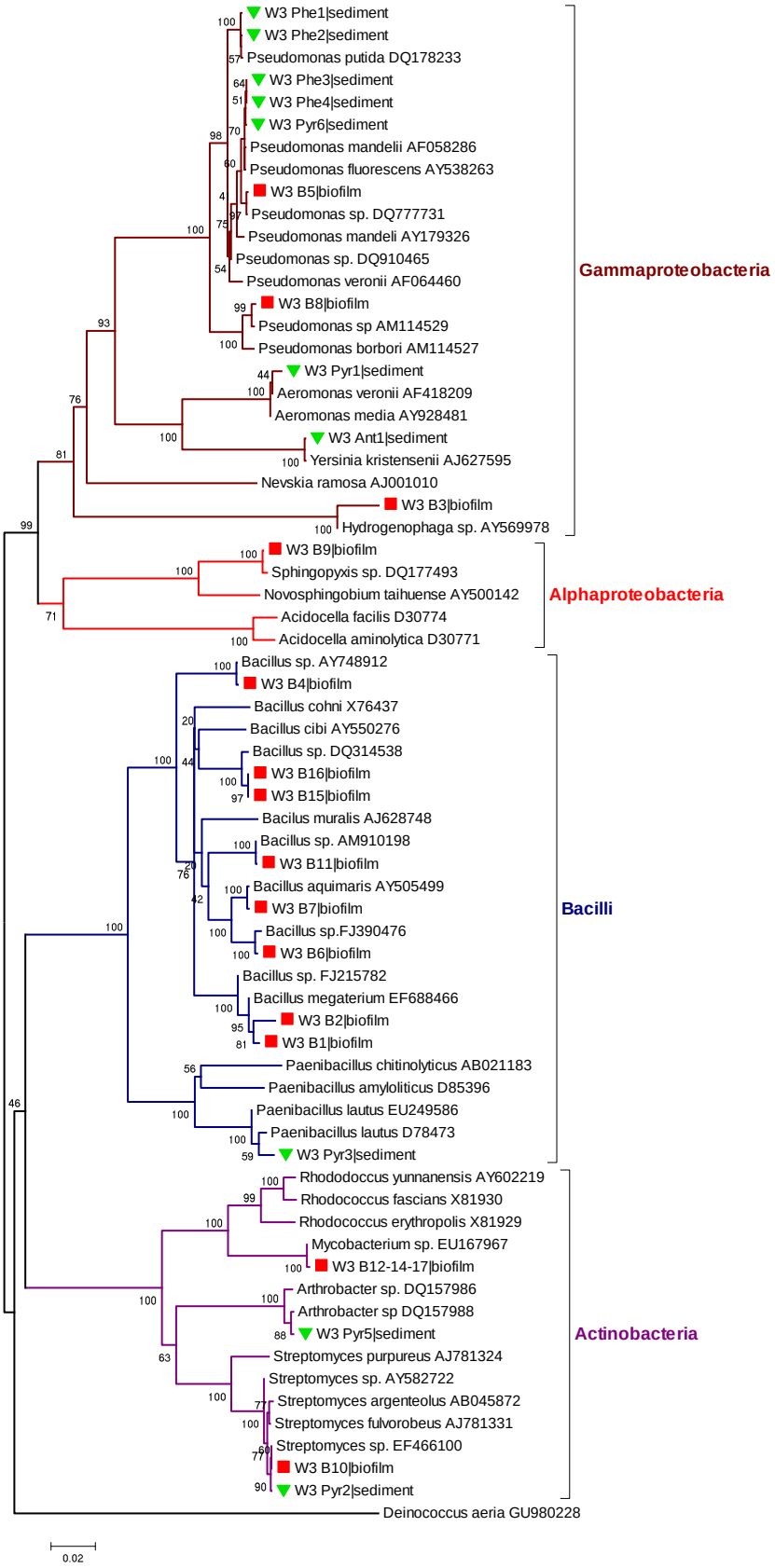


Figure 26. Phylogenetic positions of isolates from W3 based on neighbour joining clustering after multiple alignment (1450 bp) of the 16S rRNA gene sequences. Isolates from soil from Waldau 3 (inverted green triangles) and from biofilm (red squares). Branch colour denotes class-level. *Deinococcus aeria* was used as outgroup.

A detailed phylogenetic tree was constructed only with the genus *Pseudomonas* (Figure 27) because the strain W3 B8 presented low similarity with other *Pseudomonas* species and raises the question of being a new species. The closest species found was *Pseudomonas borbori*, 99.4% similarity (sequence of the fragment 16SrRNA with 1450 bp). Further analyses are necessary to verify if this strain is indeed a member of the *Pseudomonas borbori* species or is in fact a novel one.

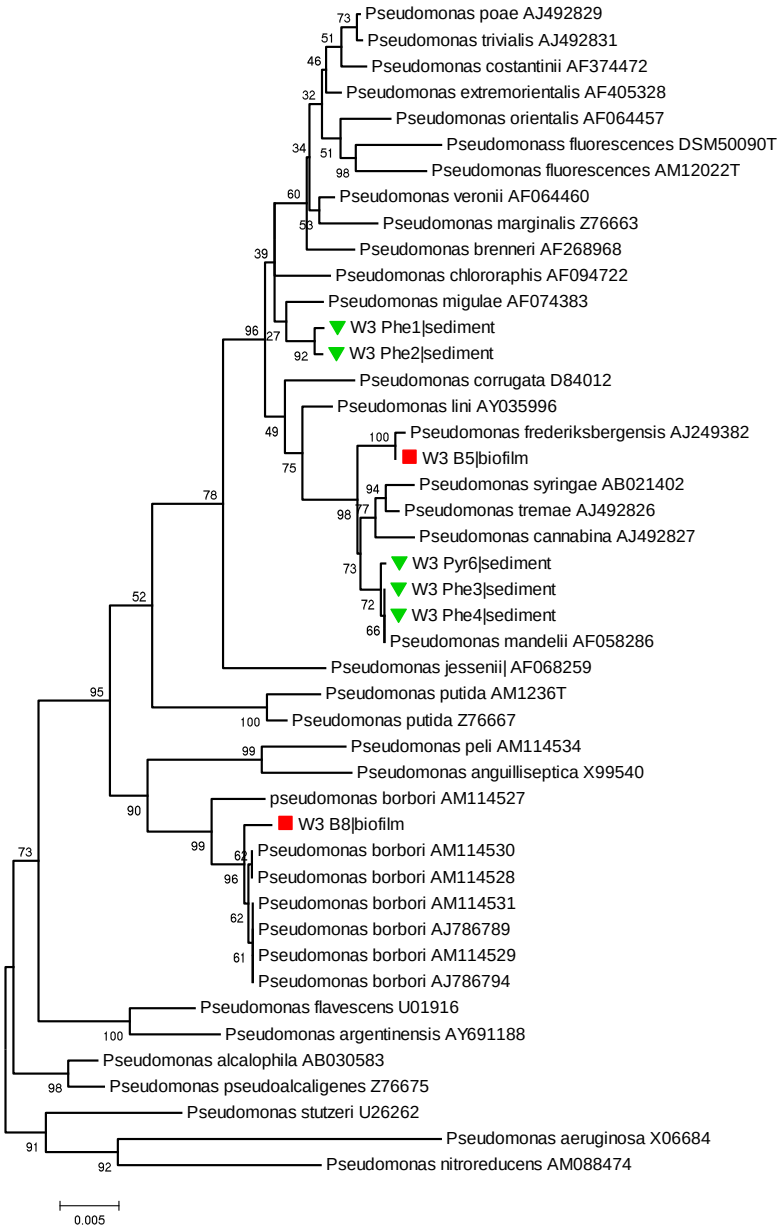


Figure 27. *Pseudomonas* phylogenetic positions based on neighbour joining clustering after multiple alignment (1450 bp) of the 16S rRNA gene sequences of the isolates from W3 sediment (inverted green triangles) and biofilm (red squares).

3.3.2 Degradation results from microcosm W3

This experiment proved that artificial communities are not as efficient as the native community from the sediment. Only fluorene and phenanthrene from the LMW PAH were degraded. Most importantly, the community constructed just with sediment isolates was not able to degrade any PAH, not even LMW PAHs. The communities “Biofilm” and “Mix” presented the same pattern of degradation (Figure 28).

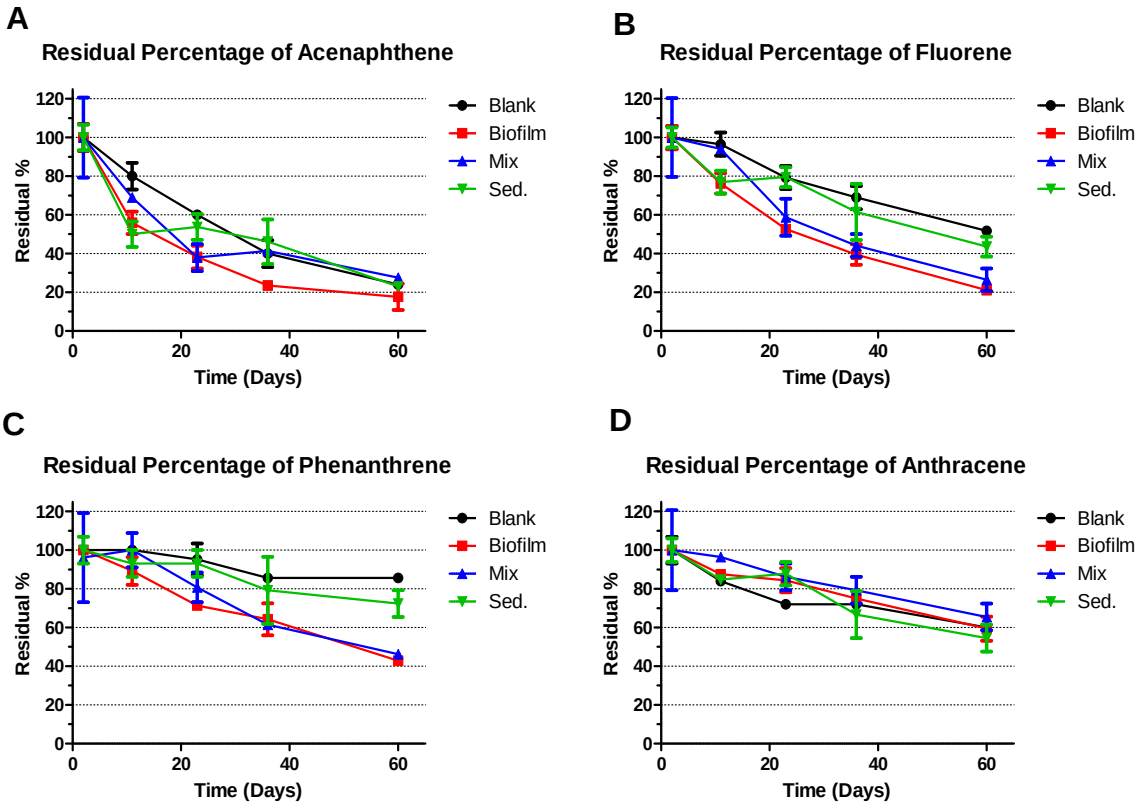


Figure 28. Degradation curve of LMW PAHs from microcosm W3. (A) acenaphthene, (B) fluorene, (C) phenanthrene and (D) fluoranthene.

Of all the HMW PAHs tested, a slight, but significant decrease in the amount of fluoranthene was noted by the communities of microcosms “Biofilm” and “Mix” within 60 days was observed, some examples are shown below in Figure 29 (ANOVA $p<0.05$, see supplementary material section 7.6.3 tables S35 – S39).

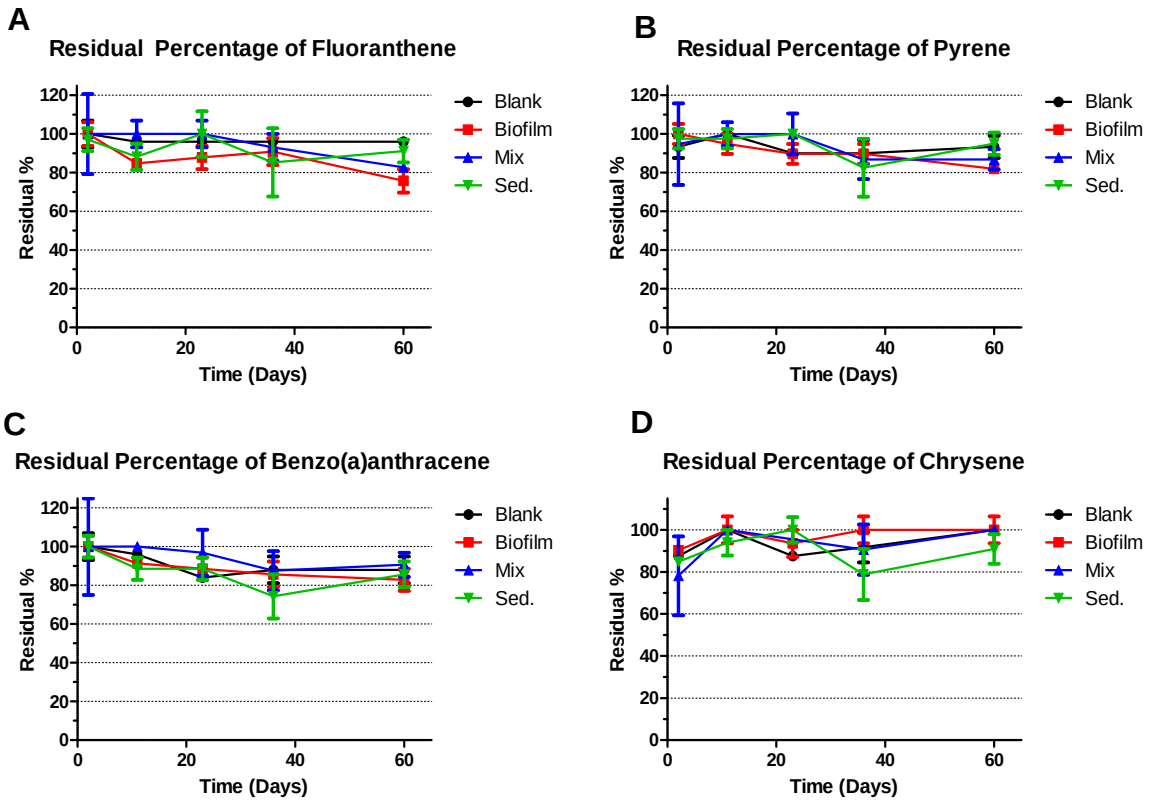


Figure 29. Degradation curve of HMW PAHs from microcosm W3. (A) fluoranthene, (B) pyrene, (C) benz(a)anthracene and (D) chrysene.

3.3.3 Communities from Microcosm W3

According to the results of degradation, where the curve of degradation from the microcosm “Biofilm” and “Mix” presented the same pattern, the SSCP gel also showed very similar pattern, as can be seen in Figure 30. Only 2 bands were different in the community “Mix” and “Biofilm” (blue arrows), one present in the first, and other in the second community. Unfortunately, both were very faint and the amount of DNA present was not enough to be amplified and sequenced. Another observation is that *Pseudomonas* sp. produced only a slight band from the community “Biofilm”, while the correspondent band was very strong from the community “Mix” as it did from the sample “Sed.”, indicating that the *Pseudomonas* species isolated from the sediment was more fit in the community than the *Pseudomonas* species isolated from the biofilm. Figure 31 shows a phylogenetic tree comparing the sequences obtained from the SSCP bands from Microcosm W3 and

the isolates from the sediment and biofilm W3. It illustrates which isolate yielded which SSCP band. In summary, microcosm experiment 3 proved that the best approach to isolate bacteria was to isolate them directly from the biofilm where a community was formed under selective pressure from the PAH.

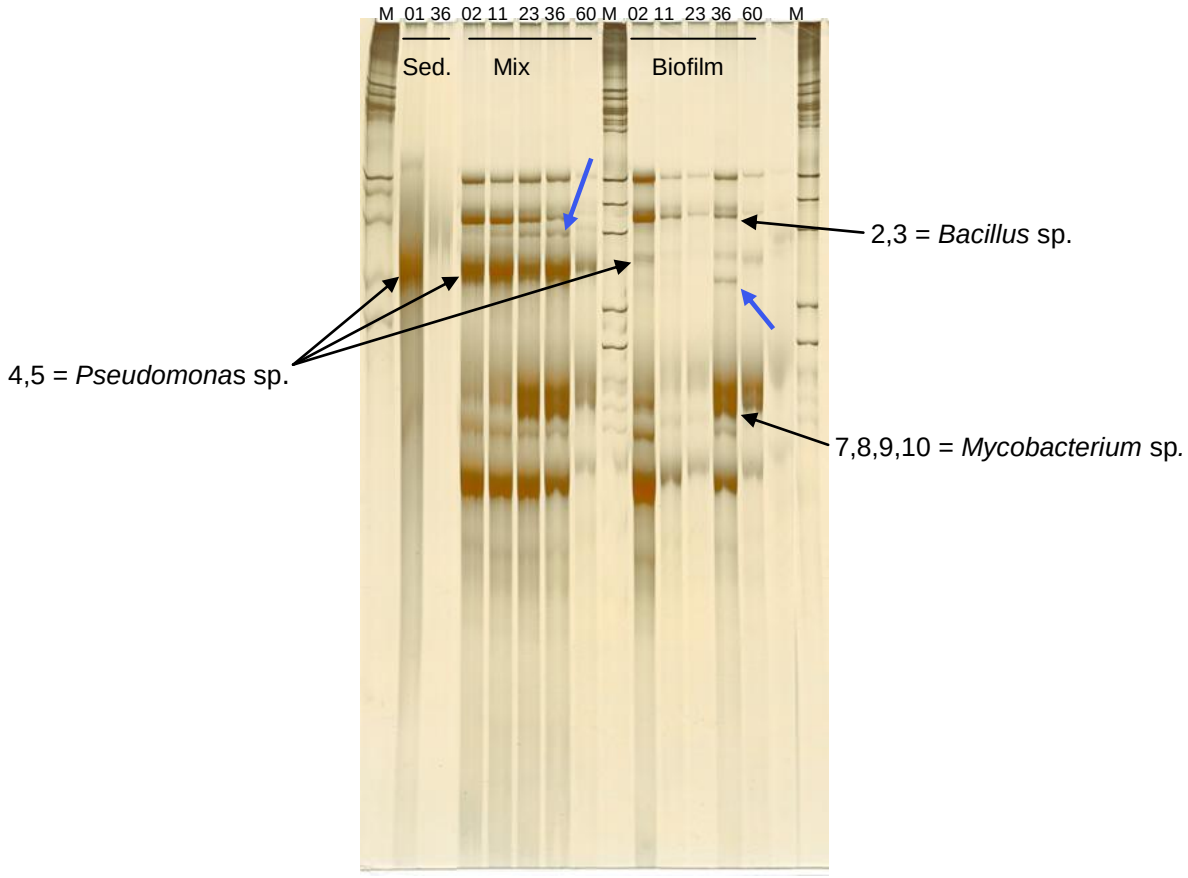


Figure 30. SSCP gel of the communities from microcosm W3. At the top, numbers indicate day of sampling and M is the position of the marker. Number and name beside the bands indicate the band used for sequencing and the respective genera. Bands at the same height on this gel produced similar sequences.

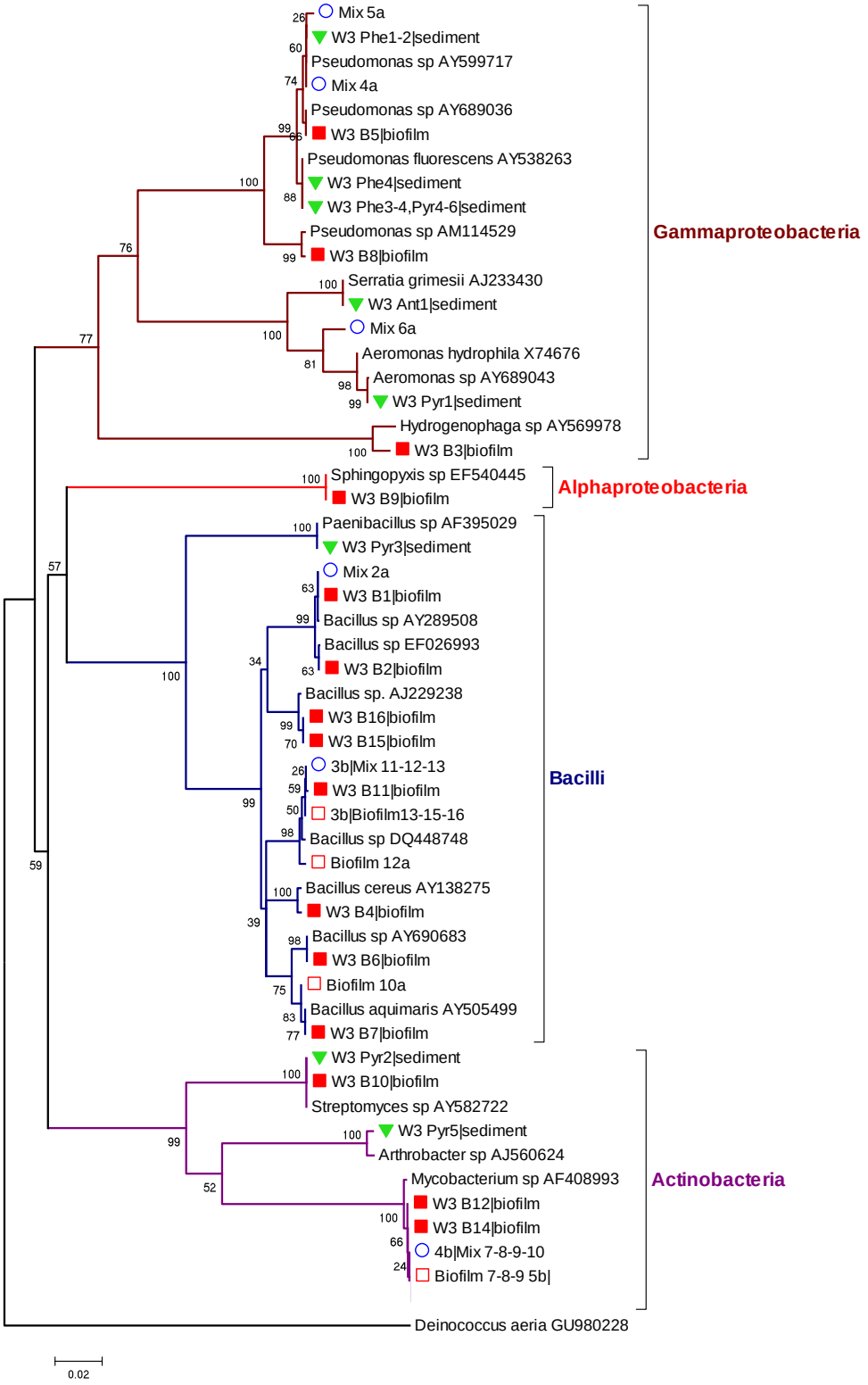


Figure 31. Phylogenetic tree with the isolates from W3, sediment (green inverted triangles) and biofilm (red squares) and representative SSCP bands from Microcosm W3 (open signs: red squares represent "Biofilm" and blue circles bands extracted from "Mix"). Branch colour denotes class-level. *Deinococcus aeria* was used as outgroup.

3.4 Microcosm 4: Artificial community, different conditions

In this microcosm experiment bacteria isolated from different sources were used to inoculate microcosms filled with 2 different media without a carbon source (M9 and BH), and 3 with BH medium supplemented with glucose, yeast extract or peptone.

Isolates from W3 (sediment and biofilm) are described in section 3.3.1, Table 5. Isolates from biofilm Abtsdorf B and C are described in section 3.2, and isolates from Rio Pinheiros belonged to the genera: *Pseudomonas* (3 isolates), *Ochrobactrum* (1 isolate) and *Kaistia* (1 isolate).

It was expected that the 52 isolates inoculated under the different conditions would be selected by nutritional condition and by the PAH crystals themselves, resulting in a community with reduced number of members than in the beginning, but still diverse and active. Surprisingly, no degradation occurred and communities could not be established. Figure 32 presents two graphs that exemplify the degradation pattern overlapping with the blank (A and B) and the SSCP gel obtained with some of the samples from different time points. The SSCP pattern of the community structure from the microcosms constructed with BH media supplemented with peptone is not shown because it is similar to the microcosm constructed with BH supplemented with glucose and yeast extract.

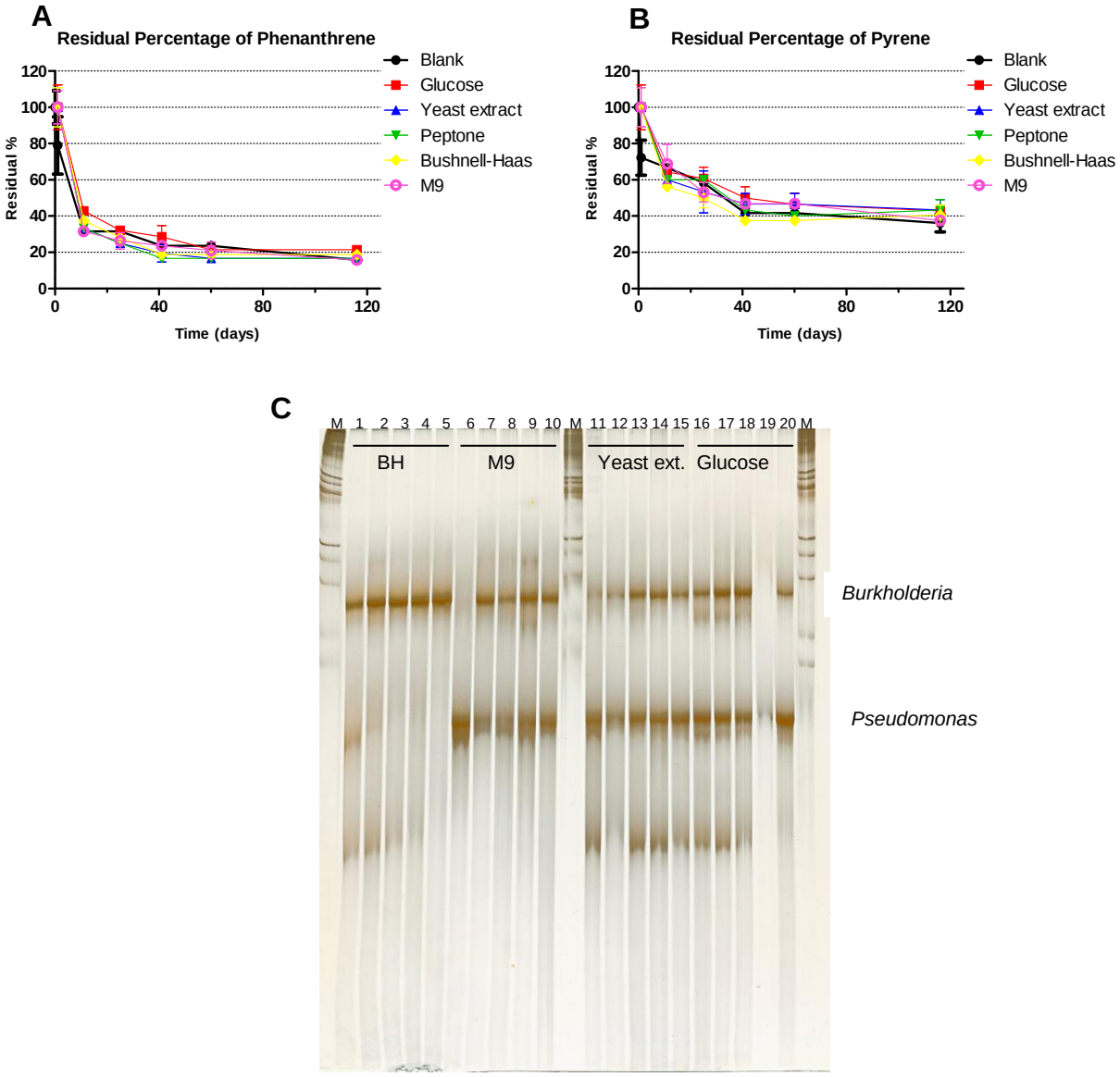


Figure 32. Amount of (A) phenanthrene and (B) pyrene measured from microcosm experiment 4. (C) SSCP gel of microcosm 4: artificial communities at different time points. Lanes M = marker; lanes 1 to 5, microcosm BH days 1, 11, 25, 41 and 60, respectively; lanes 6 to 10 microcosms M9 days 1, 11, 25, 41 and 60, respectively; lanes 11 to 15 microcosm with medium BH enriched with yeast extract days 1, 11, 25, 41 and 60, respectively; and lanes 16 to 20 microcosm with medium BH enriched with glucose days 1, 11, 25, 41 and 60, respectively.

In all microcosms the presence of *Burkholderia* sp. was detected and the presence of *Pseudomonas* sp. was also noted, except the microcosm with Bushnell Haas media. These two bacteria remained on the slides with PAH crystals during the whole course of the experiment. Finally, mixing isolates from different sources was not a good option and could not create multispecies and functional communities.

3.5 Microcosm 5: Waldau A - F

After trying to construct artificial communities with various isolates, it was concluded that they are not as efficient as the intrinsic communities from the sediment and/or soil. Thus it was decided to perform more studies using sediment as inoculum and further samples from Waldau was collected. This site was chosen because the best PAH degradation results were obtained with microcosm W3.

The new collection of samples from Waldau was performed 17 months after the first sampling and it was confirmed that the bacteria from these sites were still active despite the difference between the weather conditions comparing the previous sampling. The bacteria from this site could degrade anthracene, phenanthrene, fluoranthene and pyrene (Figure 33). Six PAHs were used in this microcosm, naphthalene was lost during drying of the sample and chrysene was not significantly degraded (data not shown). From all the sites (A, B, C, D, E and F), site C (Wal.C) was selected for further studies because this community was the fastest at degrading the PAHs, showing degradation already within 10 days.

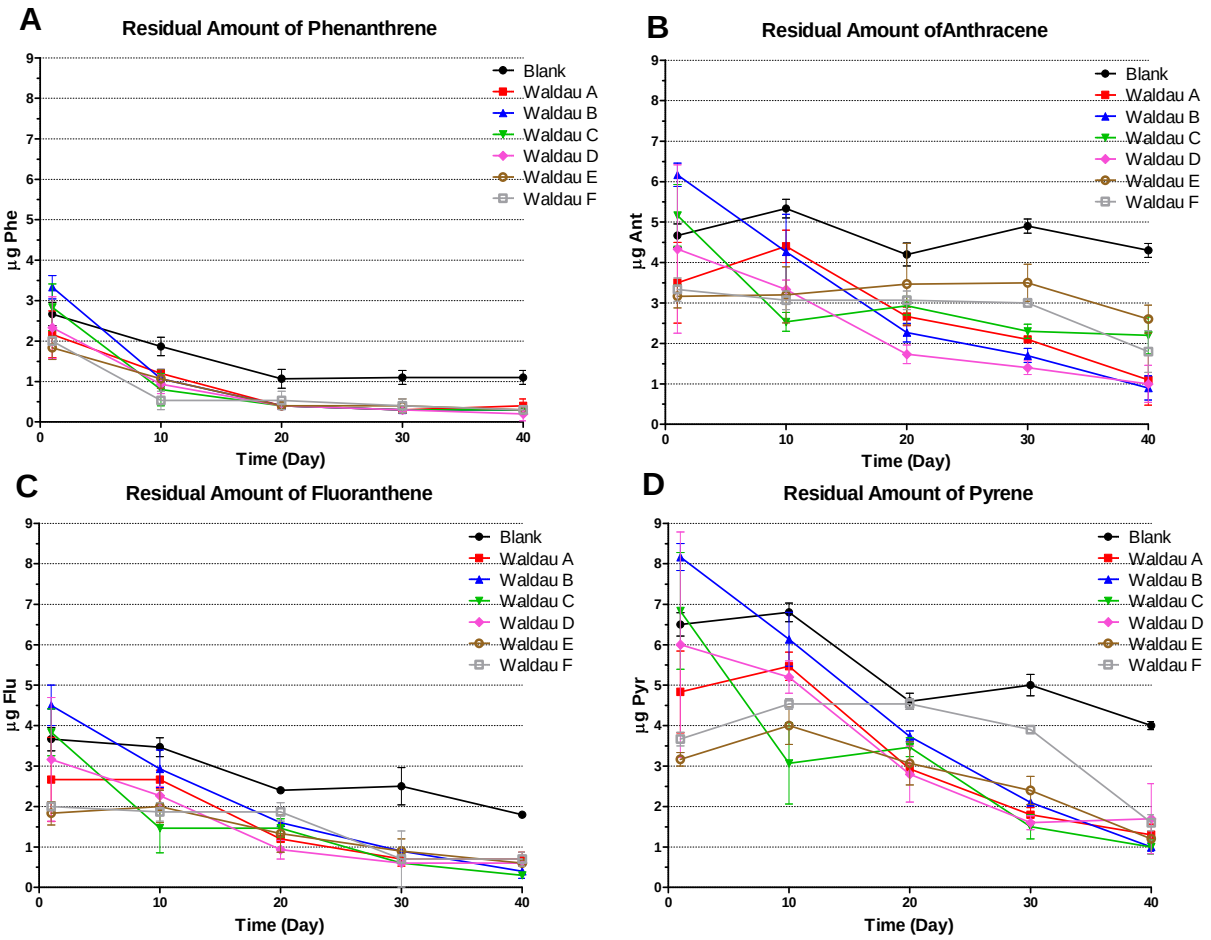


Figure 33. Residual amount of PAH in microcosm 5. (A) phenanthrene, (B) anthracene, (C) fluoranthene and (D) pyrene.

3.6 Microcosms 6: Transfer of slides to sterile microcosms

This experiment was divided in two parts, as shown in the scheme below. The PAHs used in these microcosm experiments were anthracene, phenanthrene, fluoranthene and pyrene.

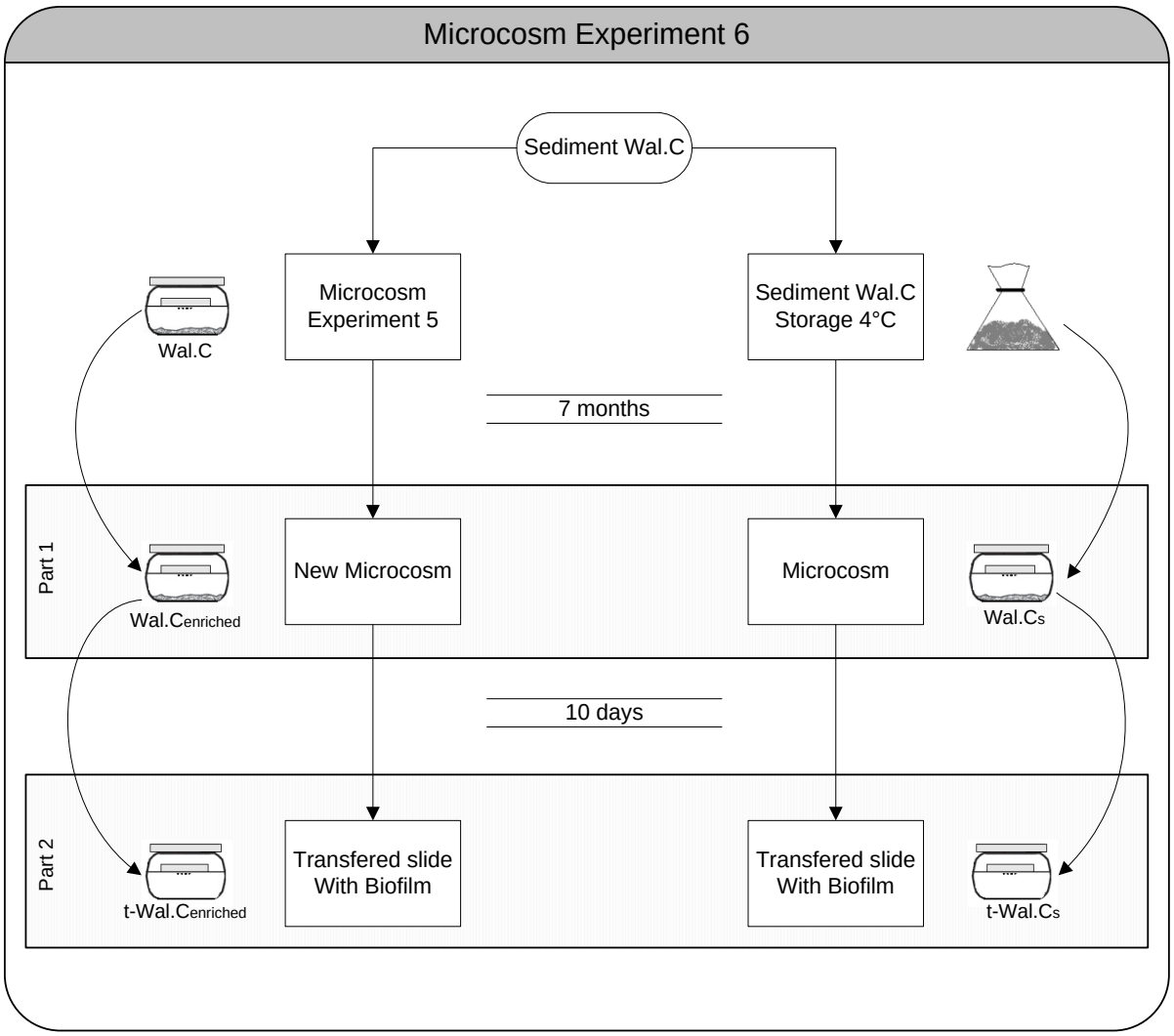


Figure 34. Experiment design of microcosm experiment 6.

3.6.1 Microcosms 6: Part 1

There was no degradation detected in the microcosm inoculated with sediment Wal.C_s as well with the sediment Wal.$C_{enriched}$, as can be seen in Figure 35.

The communities that colonized the slides with PAHs in the microcosms Wal.C_s and Wal.$C_{enriched}$, although shared some members, were in general very different from each other as can be seen in the SSCP gel (Figure 36A) and it was confirmed by the nMDS plot using the semi-quantitative analysis of the SSCP gel (Figure 36B).

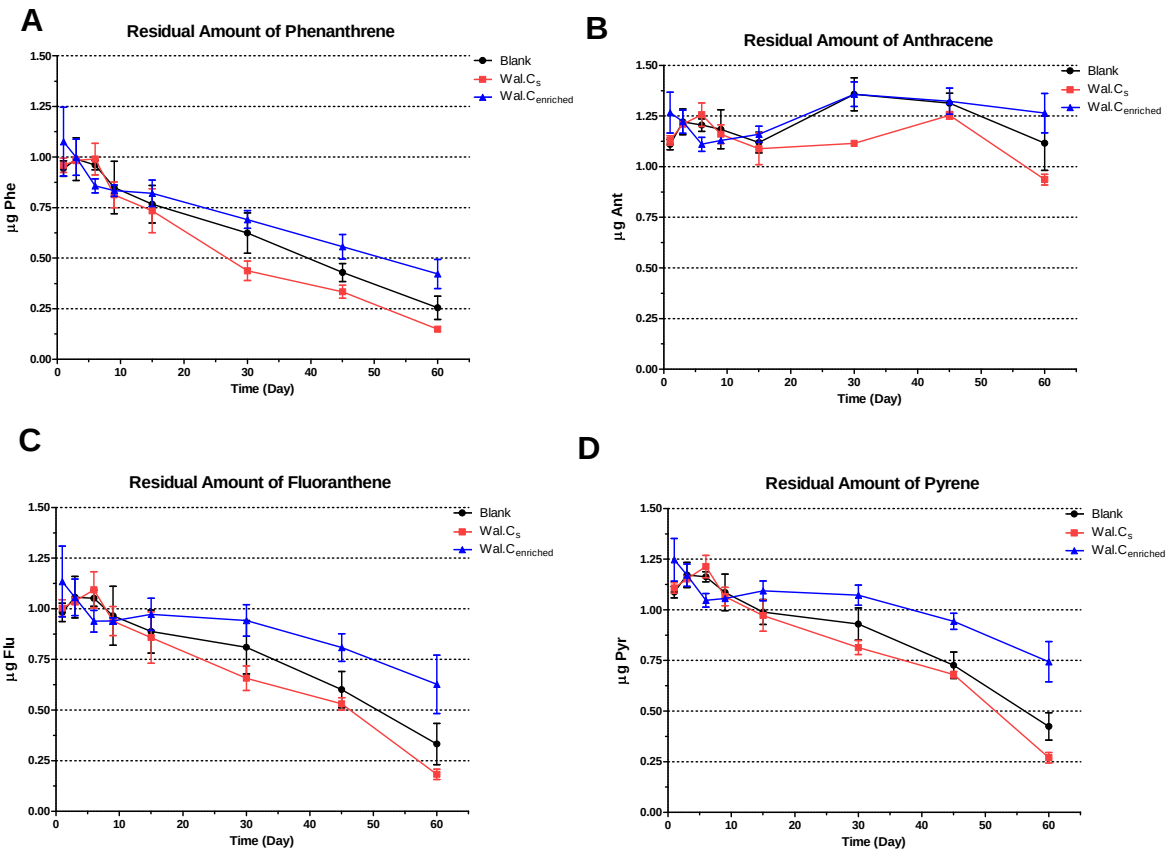


Figure 35. Residual amount of PAHs from microcosm Wal.C$_S$ and Wal.C$_{enriched}$. (A) phenanthrene, (B) anthracene, (C) fluoranthene and (D) pyrene.

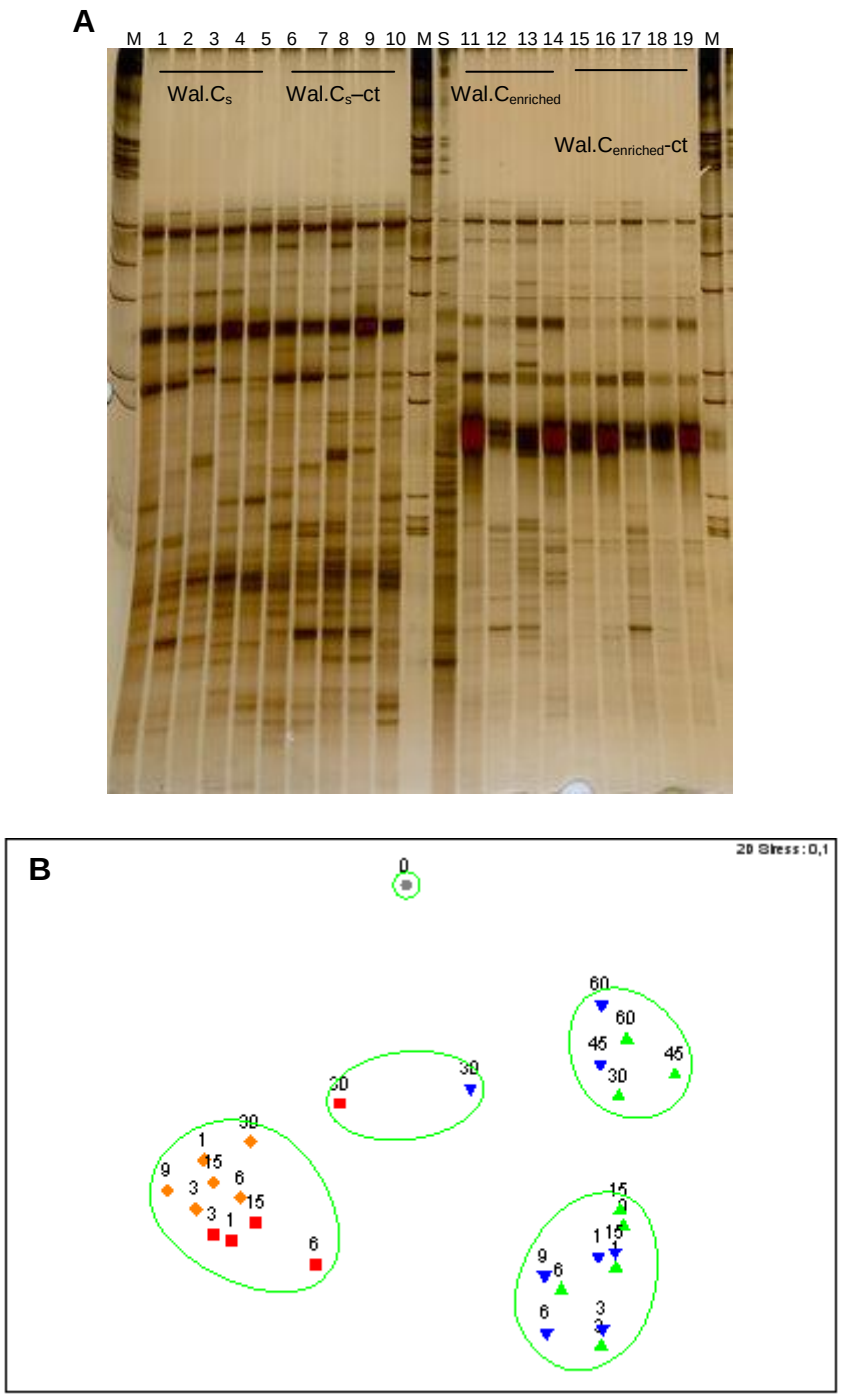


Figure 36. (A) SSCP gel from the microcosms Wal.C_s and Wal.$C_{enriched}$. Lanes M = marker; S = WC soil; lanes 1 to 5, Wal.C_s days 1, 3, 6, 9 and 15, respectively; lanes 6 to 10 Wal.C_s-control days 1, 3, 6, 9 and 15, respectively; lanes 11 to 14 Wal.$C_{enriched}$ days 1, 3, 6, and 15 respectively; and lanes 15 to 19 Wal.$C_{enriched}$-control days 1, 3, 6, 9 and 15 respectively. (B) nMDS plot of samples from Wal.C_s (green triangles), Wal.Cs-control (inverted blue triangles), Wal.$C_{enriched}$ (red squares), Wal.$C_{enriched}$-control (orange diamonds), and sediment Wal.C (gray circle), done based on semi-quantitative analysis of SSCP bands. The numbers represent time in days. Green circles around the symbols represent 65% of similarity.

The SSCP profile of the control slides without PAHs were in general 65% similar to the community attached to the PAHs, indicating that the formation of the biofilm is not completely dependent upon the substrate, but it is influenced by the intrinsic capability of the strains present in the inoculums to attach to solid surfaces. Nevertheless, the development of the biofilm and its metabolic activities is certainly influenced by the substrate to which it is exposed, as observed in the analysis of the biofilm formed in PAH single crystals in the microcosm experiment 1 Part 2 (see section 3.1.2).

3.6.2 Microcosms 6: Part 2

Slides from the previous microcosm (microcosms 6: Part 1) were transferred to new microcosms after 10 days of incubation.

Surprisingly, when the slides with biofilm from the microcosm Wal.C_s were transferred to a new, sterile microcosm; the bacteria were not just able to colonize the new PAH droplets, but they recovered the capacity to degrade the pollutants. However, the community transferred from Wal.$C_{enriched}$ did not show such ability (Figure 37) (ANOVA $p<0.05$, see supplementary material section 7.6.5 tables S45–S48).

Analysis of the sequences obtained from the SSCP bands from microcosm Wal.C_s, Wal.$C_{enriched}$, t-Wal.C_s and t-Wal.$C_{enriched}$, revealed a high prevalence of bacteria from the *Proteobacteria* phylum, and presence of *Actinobacteria* (Figure 38). The only sequence that correlates with isolates is from the genus *Mycobacterium*, proving that methods depending upon culture do not directly reflect the real picture of what happens under environmental conditions.

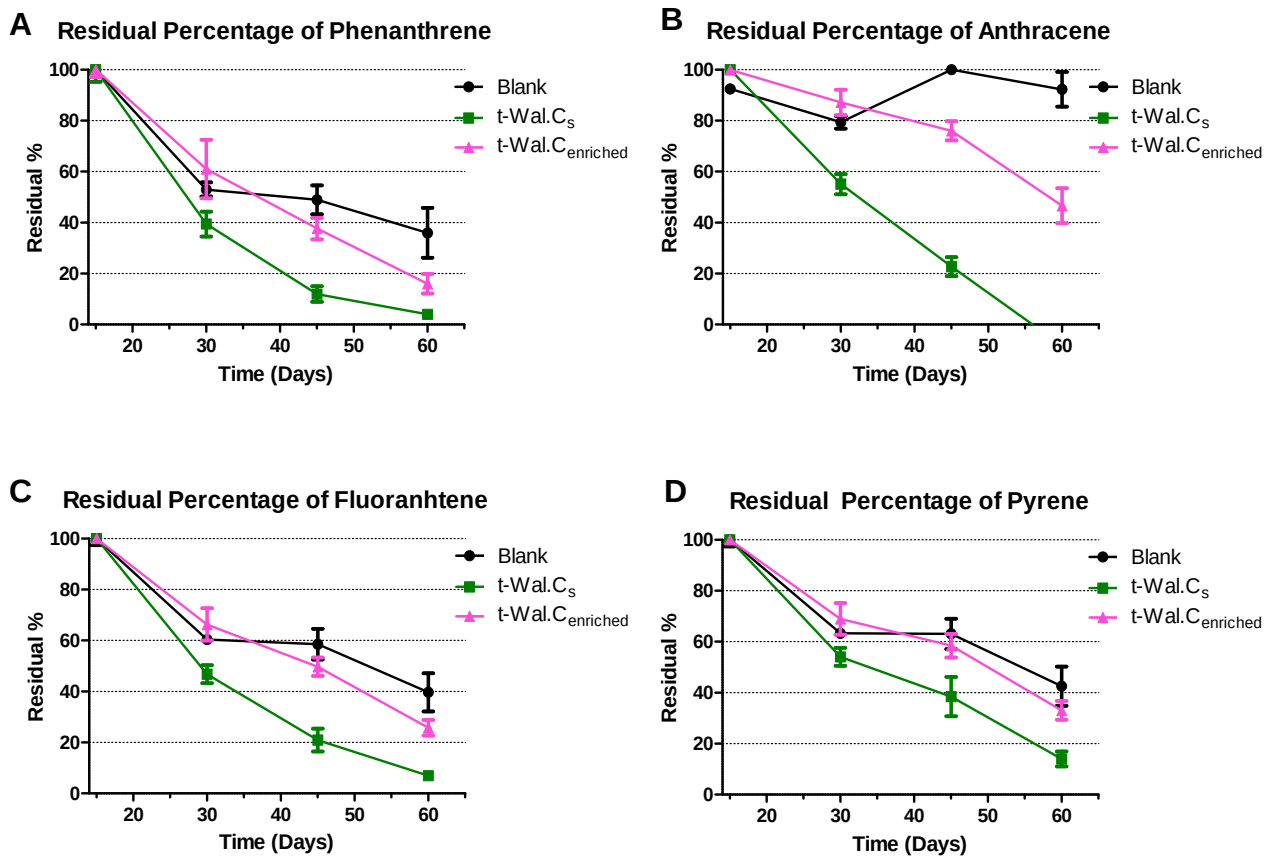


Figure 37. Residual amount of PAHs from microcosm t-Wal.C$_s$ and t-Wal.C$_{enriched}$. (A) phenanthrene, (B) anthracene, (C) fluoranthene and (D) pyrene.

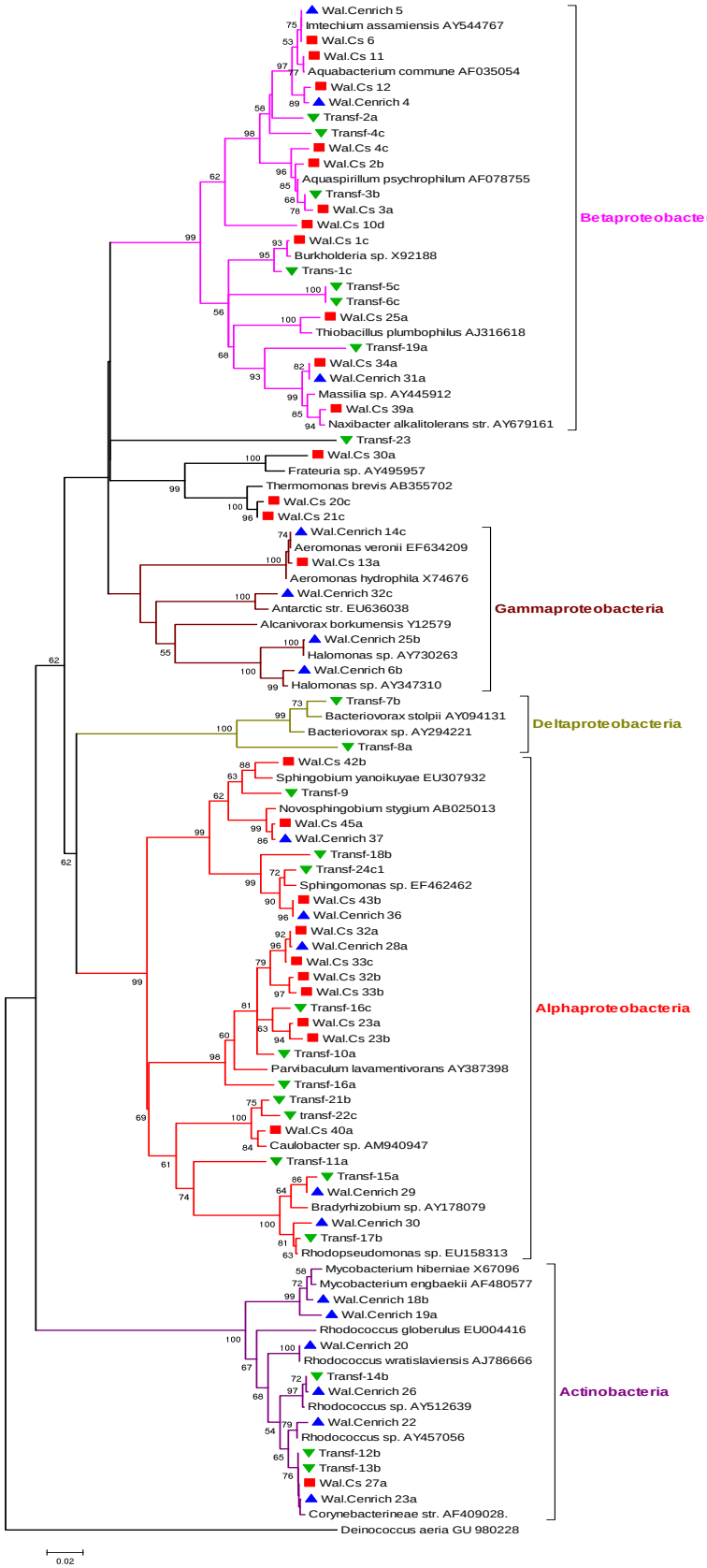


Figure 38. Phylogenetic positions of SSCP bands from microcosms Wal.C$_s$, Wal.C$_{enriched}$, t-Wal.C$_s$ and t-Wal.C$_{enriched}$ based on neighbour joining clustering after multiple alignment (360 bp) of the 16S rRNA gene sequences. Branch colour denotes class-level. *Deinococcus aeria* was used as outgroup

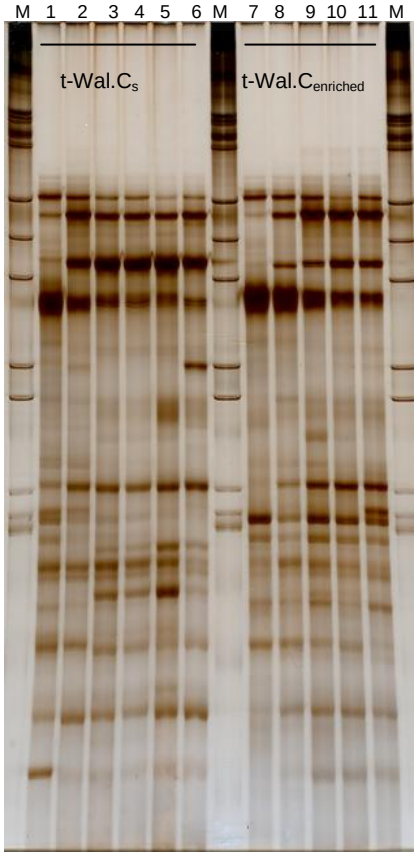


Figure 39. SSCP gel with samples from t- Wal.C_s and t-Wal.$C_{enriched}$. Lanes M = marker; lanes 1 to 6 t-Wal.C_s days 6, 15, 30, 30, 45, 60 respectively; lanes 7 to 11 t-Wal.$C_{enriched}$ days 6, 15, 30, 45, 60 respectively.

The following plots show that aging of the microcosm and contact with the PAH crystals affects the native population of the sediment; like that the SSCP profile of Wal.C_s is different from Wal.$C_{enriched}$. However when the community formed on the PAH of each of this microcosm are transferred to a new sterile microcosm, they became very similar (Figure 40 and 41). nMDS plots were constructed analyzing the results from different methods, one SSCP, which was used in this entire work; and other T-RFLP, also a culture-independent method. The analysis was made to prove that the semi-quantitative analysis of the SSCP bands is a valid method for community evaluation since both methods generated very similar nMDS plot and UPGMA cluster.

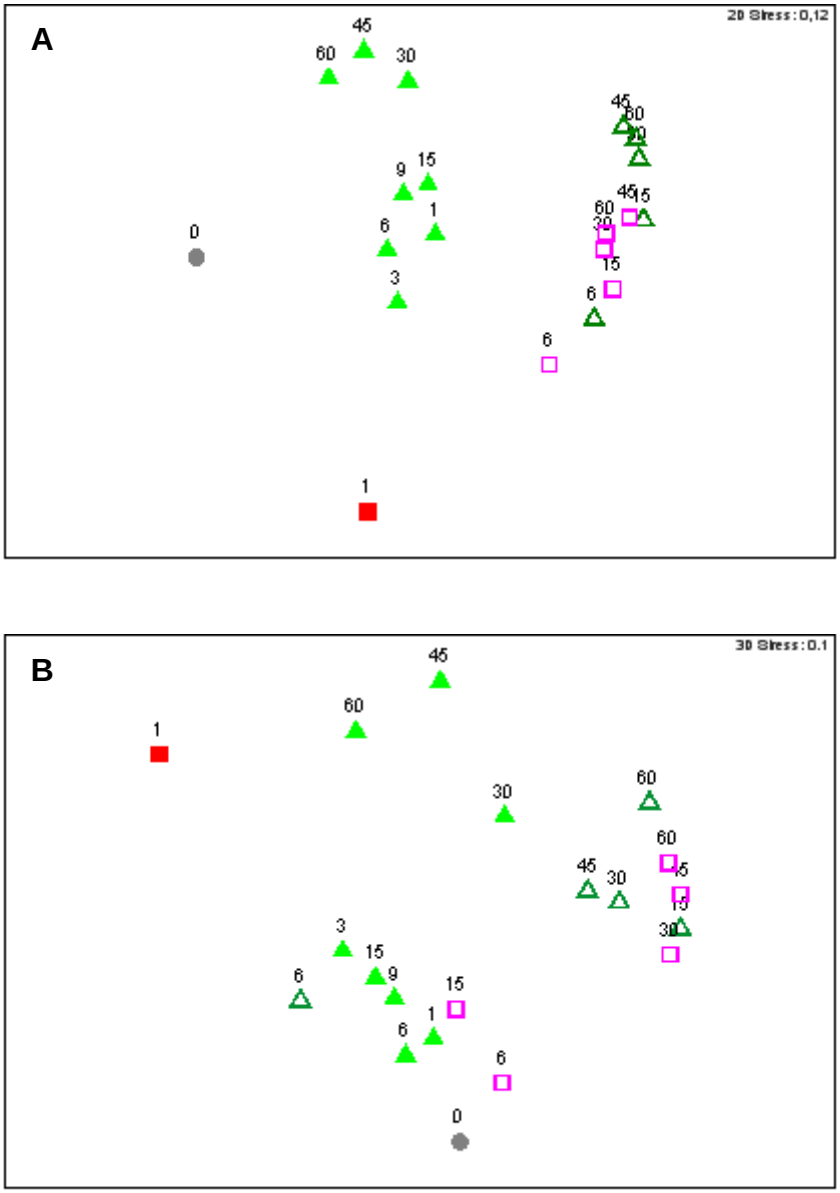


Figure 40. Microcosm 6; nMDS plot performed using SSCP and T-RFLP data from Wal.C_s (green triangles), Wal.$C_{enriched}$ (red square), t-Wal.C_s (open green triangles), t-Wal.$C_{enriched}$ (open pink squares), and sediment Wal.C (gray circle), and the Bray Curtis similarity algorithm. (A) Done based on semi-quantitative analysis of SSCP bands. (B) Done based on T-RFLP data. The numbers represent time-points in days.

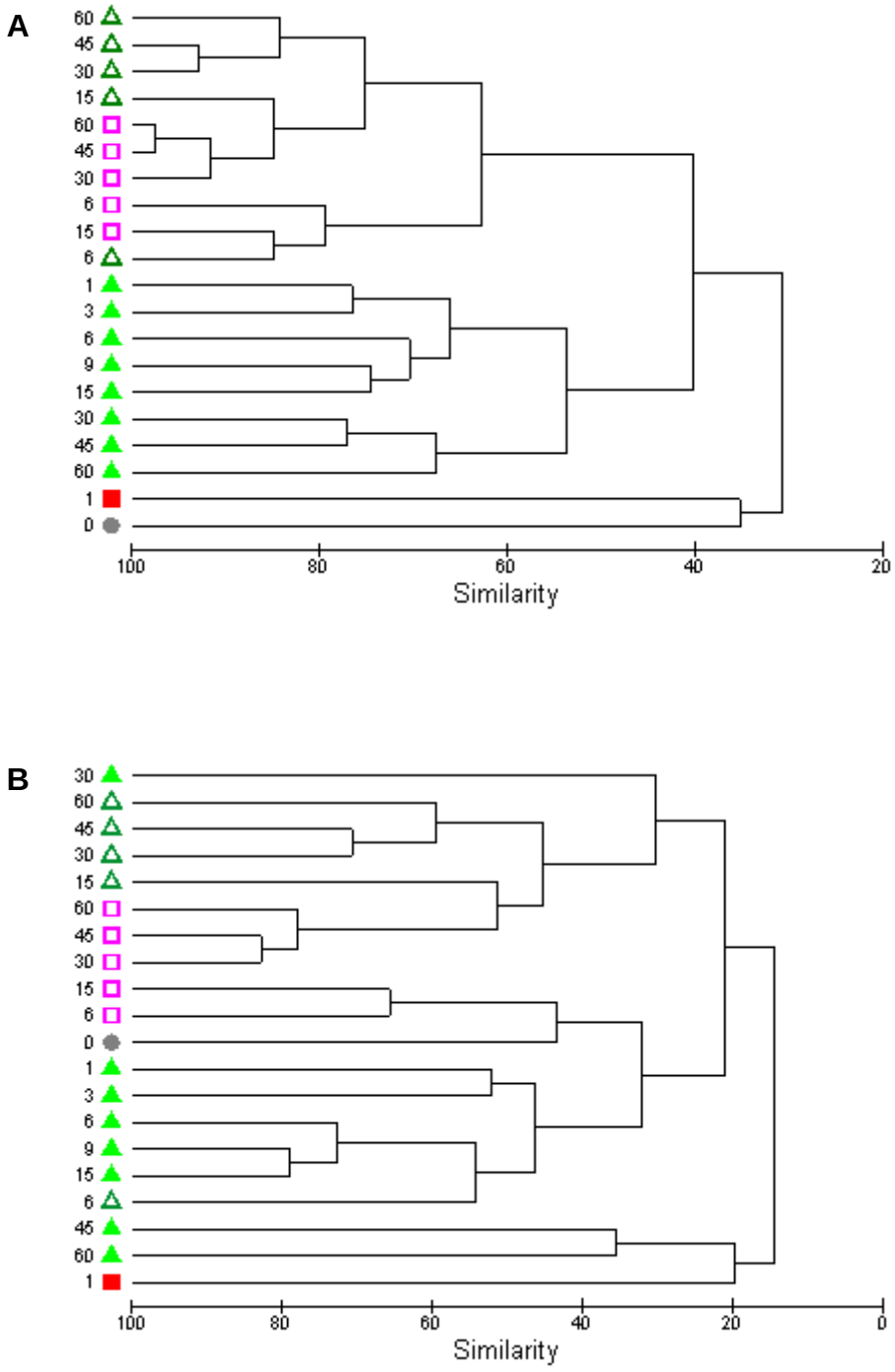


Figure 41. Microcosm 6; UPGMA cluster analysis performed performed using SSCP and T-RFLP data from Wal.C_s (green triangles), Wal.$C_{enriched}$ (red square), t-Wal.C_s (open green triangles), t-Wal.$C_{enriched}$ (open pink squares), and sediment Wal.C (gray circle), and the Bray Curtis similarity algorithm. (A) Done based on semi-quantitative analysis of SSCP bands. (B) Done based on T-RFLP data. The numbers represent time-points in days.

3.7 Bacterial enrichment experiments

A third attempt to isolate strains was made employing the method of enrichment. With this approach the result was again different from the others. Seven different genera were identified: *Paracoccus, Citrobacter, Pseudomonas, Stenotrophomonas, Sphingobacterium, Ochrobactrum* and *Brevundimonas*.

The only genus that correlates with the previous isolates, from soil and biofilm, was *Pseudomonas*. This result leads to the conclusion that the genus *Pseudomonas* has a high advantage under laboratory conditions comparing to other bacteria and it is possible to be isolated under the most diverse approaches.

4 Discussion

4.1 Degradation

LMW PAHs are catabolised more rapidly by microorganisms than HMW PAHs (Cerniglia and Heitkamp, 1989). As Molina and co-workers (Molina *et al.*, 1999) stated, “if the catabolic enzymes are less specific, metabolism of HMW PAHs may be induced by more readily degradable substrates, such as the two- and three-ring PAHs. Because HMW PAHs solubilise slowly, the enzyme system necessary to initiate transformation of these compounds may be activated by a similar substrate with a faster solubilisation rate such as the LMW PAHs”. Accordingly, in the present work LMW PAHs were maximally degraded within the first 30 days where their abundance only slightly decreased for the remainder of the experiment, whereas HMW PAHs required 90 days to reach this same level of degradation, three times longer than the LMW PAHs. One exception to this rule was anthracene. This compound continued being degraded after phenanthrene was mostly consumed, although both have the same molecular weight (178.2 g). There is some discussion about the difference on degradation rates of phenanthrene and anthracene and the major reason for this is probably because phenanthrene is about 20 times more soluble than anthracene; phenanthrene 1.3 mg l^{-1} and anthracene 0.07 mg l^{-1} (Mueller *et al*. 1989; Cerniglia, 1992). Also, the structure of anthracene is without a K region (Phale *et al*., 2007) and so is more difficult to metabolize, whether because of its chemical stability or due to it having a different enzymatic binding site.

The pattern of degradation of HMW PAHs in this work was complex and presented fluctuations during the first month of incubation. Particularly, the amount of benzo(g,h,i)perylene (data not shown) and indeno(1,2,3-*c,d*)perylene presented extreme variations that were noted either by fluorescent detector or PDA detector. This might have occurred due to metabolites generated by the catalysis of PAHs, which interfere with the detection of these compounds.

In summary, the majority of the tested PAHs could be degraded by communities from sediment and soil even those from locations where there is no

reported PAH contamination. Thus, this show that there is great potential of bacterial communities in the field for bioremediation.

Naphthalene is the smallest PAH used in this work, it sublimes at room temperature, and therefore it is lost during the concentration of the sample, when they are exposed to a stream of nitrogen. Even though, this compound was initially used in most of the microcosms, however not subsequently measured, but to be utilized as a starter of the process due to the fact that naphthalene degrading enzymes can present relaxed substrate specificity, like this they can be employed either in naphthalene or phenanthrene degradation (Kiyohara *et al*., 1994; Balashova *et al*., 2001). Foght *et al*. (1990) suggested an existent hierarchy on PAH degradation: bacteria strains able to mineralize monocyclic and dicyclic aromatics do not necessarily degrade tricyclic aromatics substrates, otherwise microorganisms that degrade tricyclic aromatic hydrocarbons are able to degrade less complex compounds. A good example of this, is the work of Johnsen and colleagues (Johnsen *et al.*, 2007), who characterized degraders from a PAH contaminated soil then fertilized it for 4 years with NPK and isolated five different *Mycobacterium* species and one *Arthrobacter* species. The evaluation of the capacity of these bacteria to grow using phenanthrene, pyrene and fluoranthene demonstrated that *Arthrobacter* could grew only on phenanthrene, while all pyrene-degraders grow on phenanthrene as well. Pyrene is probably shuttled into the phenanthrene pathway through formation of 3,4-dihydroxyphenanthrene, going then into the phenanthrene *o*-phthalate pathway, confirming the hypothesis that microorganisms that degrade tricyclic aromatic hydrocarbons are able to degrade less complex compounds. Furthermore, the degraders of fluoranthene could neither grow on phenanthrene nor pyrene, indicating metabolism via different compounds not used in the other degradation pathways and consequently requiring different enzymatic machinery (Figures 3, 4 and 5).

4.2 Communities

4.2.1 Waldau 3

The evaluation of the communities that colonized the slides with single crystals of PAHs on microcosm experiment 1 (Part 2) inoculated with the soil W3 nicely proved that a consortia is highly influenced by substrate availability. Although the samples from the bands extracted from the SSCP gels did not provide sufficient amount of DNA to generate sequences, probably due to aging of the microcosm; it is clear that each LMW PAH selected for different strains, as well as 2 of the smaller HMW PAHs that only contain 4 rings, fluoranthene and pyrene; but more complex HMW PAH, with similar chemical structure presented very similar pattern of SSCP bands (Figure 20 and 21), indicating that the community which colonized these crystals comprised the same community structure. To my knowledge this is probably the first study that utilized such complex compounds and compared community structure where it was possible to show that a single microcosm, with different substrates could simultaneously select for different yet diverse communities.

Despite this observation, it was proposed to create a simpler artificial community to study where it could be possible to maintain and manipulate under laboratory conditions, but still capable of metabolizing mixtures of PAHs. Therefore, bacteria were isolated from sediment of W3 and also from the biofilm formed on the PAH crystals in the microcosm inoculated with this same soil sample. Both sources provided isolates from the genera *Pseudomonas* and *Streptomyces*, as expected because these two genera are largely found in soil and sediments (Ventura *et al*., 2007; Peix *et al.*, 2009). On the contrary, the other strains isolated either directly from the soil or from the biofilm were quite different between each other belonging mainly to the phyla *Proteobacteria* and *Firmicutes*.

The last attempt to select PAH degraders was performed by enrichment in liquid culture. Again, strains different from the other methods were isolated and once more the genus *Pseudomonas* was present indicating that this bacteria has advantages under laboratory conditions and this is probably the reason why so many studies are performed with the genus.

These isolation attempts confirmed the hypothesis that classical microbiological methods of isolating microorganisms (generally directly from soil/ sediment) are not robust enough to characterize the bacterial members of soil and sediments and consequently select known microorganisms that have a competitive or adaptive advantage under laboratory conditions, which may not be true under real environmental conditions. The isolates from the biofilm still underrepresented the community from the original sediment W3 (characterized by a culture-independent method – SSCP), but could select for important degraders in comparison to those isolated directly from the sediment which could not degrade any of the tested PAHs. In agreement with this, Bastiaens *et al.* (2000) compared two methods to isolate bacteria from PAH-contaminated soil and sludge samples: using shaken enrichment cultures in liquid mineral medium in which PAHs were supplied as crystals and a method in which PAH degraders were enriched on and recovered from hydrophobic membranes containing sorbed PAHs. With the first method the genus *Sphingomonas* was mainly selected, whereas the membrane method exclusively selected for *Mycobacterium* sp. Thus, different cultivation/isolation methods produced different results.

A relevant observation regarding the isolates from W3 is that all genera identified, except *Hydrogenophaga* and *Yersinia*, are reported as degraders of pollutants, especially PAHs.

Some of the isolates from W3 were similar to those from the work of Daane *et al*. (2001) who isolated strains from contaminated estuarine sediment and salt marsh rhizosphere by enrichment using either naphthalene, phenanthrene, or biphenyl as the sole source of carbon and energy. They isolated strains of *Paenibacillus*, *Arthrobacter* and *Pseudomonas*. Experiments using liquid suspensions proved the ability of the isolates to degrade naphtalene, but none of the tested isolates were able to utilize pyrene in the same liquid conditions. Nevertheless, *Paenibacillus validus* and *Arthrobacter oxydans* promoted pyrene degradation when inoculated into an aerobic marine sediment slurry containing a mixture of naphthalene, fluorene, phenanthrene, and pyrene. They concluded that, in general, the strains isolated on phenanthrene were able to utilize a greater number of PAHs than the strains isolated on naphthalene.

Another study that reported on the bacteria able to degrade PAH similar to the present work, isolated them from Elizabeth River sediments, in the USA. (Hilyard *et al*., 2008). They identified isolates and DGGE bands that belonged to the genera*, Rhodococcus, Pseudomonas, Mycobacterium* and *Novosphingobium*, among others. *Mycobacterium* and *Novosphingobium* were also found by Gauthier *et al.* (2003) and later again by Lafortune *et al*. (2009). Gauthier and co-workers selected the HMW PAHs degraders *Mycobacterium gilvum* B1, *Bacillus pumilus* B44, *Mycobacterium esteraromaticum* B21 and *Porphyrobacter* after 31 months of enrichment with HMW PAHs. After this, the same consortium was kept for 3 years frozen and a new study was performed by Lafortune *et al*. (2009), who identified more strains present such as *Novosphingobium*.

Besides *Mycobacterium, Streptomyces* is another genus from the class *Actynobacteria* isolated in the present work that is known to carry catabolic genes of lower pathways of PAH degradation, as reported by Ishiyama *et al*. (2004) that identified a strain with 99% sequence similarity to *Streptomyces resistomycificus* able to degrade salicylate and 4-hydroxybenzoate.

In the first attempt to combine the isolates from this work to create artificial communities, the microcosm inoculated only with the isolates from the sediment W3 (“Sed.”) was not able to degrade any PAH and it was also not possible to extract enough amount of DNA to proceed with deep analyses of the community, leading to a conclusion that these strains were neither able to colonize properly the PAH crystals nor to form an active consortia. The little amount of DNA extracted from this sample was loaded onto a SSCP gel where it produced only one strong band identified as *Pseudomonas* (Figure 30). It was then possible to conclude that the single genus isolated from soil W3 able to colonize PAH crystals was *Pseudomonas*, which was not very surprising because this genus is well known as being capable to degrade PAHs (Mueller *et al*., 1990; Cerniglia, 1992; Gomes *et al*., 2005; Arun, 2008).

In contrast, both communities derived from “Biofilm” and “Mix” could successfully colonize the PAH crystals and degrade fluorene and phenanthrene. The SSCP showed that the community “Biofilm” and “Mix” were very similar, indicating that bacteria coming from the biofilm were better adapted to the conditions of the

microcosms, and consequently more successful in colonizing the slides containing PAH crystals. This result confirmed the hypothesis that isolating bacteria directly from the biofilm instead of the sediment (or soil), would select strains adapted to the presence of PAHs and they would be fitter within a consortium if they had previously "co-habited" in the same biofilm.

4.2.2 Abtsdorf

A previous study performed by Macedo *et al*. (2005) showed the degradation of polychlorinated biphenyls (PCB) in microcosms with soil samples from Abtsdorf. For that reason samples from this same location were taken in the present study to compare the degradation rates of PAHs of these microorganisms with those from the community W3 which could degrade several different PAHs. Although there was degradation of different PAHs by the community from Abtsdorf site B and C, they were not as effective at degrading the array of PAHs as the community W3, which showed faster degradation and ability to metabolize a wider variety of PAHs. Incidentally, it was observed that the samples taken from the four sites at the location of Abtsdorf, behave differently between each other. As an example, the sample from the site considered non-polluted called "Clean" was not able to degrade the tested PAHs, while that from the site called "Abtsdorf B" and "Abtsdorf C" could degrade PAHs to a certain extent. The T-RFLP analysis done with these four samples (Abtsdorf B, C, 54 and Clean) showed that the difference in the degradation profile is consistent with the different community profiles.

Considering that the strains comprising the biofilm Abtsdorf B and C were able to grow on and degrade some of the PAHs, even to a lesser extent than W3, bacteria were isolated from the biofilm formed on the PAH crystals in these microcosms with the same objective as those bacteria isolated from W3: to construct an artificial community capable of metabolizing mixtures of PAHs. Bacteria from the soil were not isolated because the previous experiment with artificial communities proved that bacteria from the sediment are not likely to form a consortium whereas the opposite was observed with bacteria isolated directly from the biofilm.

The main difference observed between the isolates from Abtsdorf and W3 was that Abtsdorf was much less diverse, where only four distinct genera were found, *Bacillus*, *Burkholderia*, *Collimonas* and *Pseudomonas*. However, all of them are reported as PAH degraders (Johan *et al*., 2010; Wilson *et al*., 2003).

4.2.3 Artificial communities

With so many isolates cultured in this study, it was impossible to propose a criteria to select just a few of them to construct an artificial community. Thus we relied on the 'selection effect' defined as the effect caused by the dominance of species with particular traits affecting ecosystem processes (Loreu and Hector, 2001), as so the PAHs crystals themselves would be able to select bacteria apt enough and cooperate in a community. However, when the isolates were introduced in one single microcosm no consortium could be established, instead, only *Burkholderia* and *Pseudomonas* attached onto the PAH crystals.

What possibly happened was that the strains inoculated were metabolically similar to each other, stimulating competition rather than cooperation between them. Bell *et al*. (2005) stated: "Two principal mechanisms underlie the current understanding of how biodiversity affects ecosystem functioning, and especially those functions related to ecosystem productivity (the rate of biomass turnover). First, different species use slightly different resources. Species-rich communities are therefore more productive because more of the overall resource is used (the 'complementarity mechanism'). Second, there is variation in the magnitude of individual species' effects on ecosystem functioning. Species-rich communities are therefore more productive on average because they are more likely to contain species with a large effect on ecosystem functioning (the 'selection mechanism'). Both mechanisms predict a decelerating diversity–functioning relationship under some conditions". As such it is plausible to affirm that in the first artificial communities (microcosm experiment 3) with either 16 or 27 members, the consortia were still diverse enough to avoid substrate competition and the bacteria from these communities had previously proved to be able to form a consortium, since they were isolated from a biofilm. On the contrary, the attempt to put 52 isolates together, many

of them being from the same genera, e.g. *Burkholderia, Pseudomonas* and *Bacillus*, compromised the stability of the system and stimulated competition between strains having similar metabolic requirements.

4.2.4 Microcosm Wal.C_s; Wal.$C_{enriched}$; t- Wal.C_s and t- Wal.$C_{enriched}$

As the attempts to create artificial communities were not satisfactory, it was decided to focus on the sediment communities and how they behave with time. Therefore new samples were taken at Waldau, which had already been proven to be the most active site used in this work.

After confirming that the communities from this location were active, a new approach was used to evaluate the behaviour of the consortium. New microcosms were constructed using sediment from Wal.C stored at 4°C for seven months (Wal.C_s) which was the same sediment used in the previous microcosms experiment, where its first contact with PAHs occurred seven months earlier (Wal.$C_{enriched}$). The communities from both microcosm experiments were relatively different, Wal.C_s comprised a prevalence of species belonging to the phylum *Proteobacteria*, classes *alpha-, beta-* and *gammaproteobacteria* with just one sequence not related being closely related to *Rhodococcus*. In the microcosm Wal.$C_{enriched}$ the phylum *Proteobacteria* was reduced to much less members, where the prevailing species belonged to the classes *alpha-* and *gammaproteobacteria*. The opposite was observed with the phylum *Actinobacteria* that presented an increased number of members comparing with Wal.C_s community, with species related to *Rhodococcus* and *Mycobacterium*. Surprisingly, no bacteria from the genus *Pseudomonas* were detected in any of these microcosms.

In accordance with the present work, Hilyard and co-workers (Hilyard *et al.*, 2008) investigated the diversity of indigenous bacteria from sediments of a river able to degrade PAHs by classical selective enrichments and molecular analysis and they also found *Actinomycetes, alpha-, beta-* and *gammaproteobacteria*. Johnsen *et al.* (2002) found s*phingomonads* and an unidentified *betaproteobacteria* as responsible for the PAH degradation after the initial PAH degradation (which is a rapid degradation phase). The same group found a different picture from the same soil

after 4 years of enrichment and fertilization (Johnsen *et al.,* 2007), when they isolated only *Actinobacteria*; five species of *Mycobacterium* and one species of *Arthrobacter*. This finding correlated with those of Leys *et al.* (2005) who suggested that initial degradation of PAHs in soil is done by *Sphingomonas* and other fast-growing bacteria, whereas later, the *Sphingomonas* are outcompeted by relatively slow-growing *Mycobacterium*.

A surprising fact in the present work was that both the communities Wal.C_s and Wal.$C_{enriched}$ apparently lost the ability to metabolize the PAHs tested (Figure 35). Furthermore, those members from $WC_{enriched}$ became scarce as indicated by the low yield of DNA extracted from the slides with PAHs, leading to the conclusion that the community was not viable anymore probably due to aging of the microcosm and lack of nutrients and co-factors for enzymatic processes. However when the bacteria from these microcosms were transferred to new sterile microcosm containing a Bushnell Haas medium without a carbon source, the communities re-established in a very similar manner, but curiously only the community of Wal.C_s was able to degrade PAH. From this observation it could be proposed that this occurred because the bacteria from Wal.$C_{enriched}$ suffered from nutrient deprivation due to aging of the microcosm therefore it needed some time to recover and become active again. The members of the new communities belong to the same classes as described before *Actinobacteria, Alpha-, Beta-* and *Gammaproteobacteria*, but very interestingly, the class *Gammaproteobacteria* previously abundant in both microcosms, was reduced to only one member.

Consistent with our work, Johnsen *et al.* (2007) found several members from the phylum *Actinobacteria* when he used aged PAH-contaminated soil, especially *Mycobacterium* sp.. It was found that using inoculums of soil with its intrinsic degraders, resulted in a high survival rate of bacteria, which are still culturable after 16 weeks of incubation, whereas inoculum from liquid cultures presented a fast decline. Liquid techniques may induce genetic changes, selecting for planktonic cells, that may lose their ability to excrete extracellular polymeric substances and to attach to surfaces and form biofilms. Biofilm formation is very important for cell survival in soil because it protects them against environmental stress and predation of protozoa (Queck *et al*., 2006). Furthermore, biofilm formation directly on PAH

crystals by degraders facilitate its uptake because this reduces the constrains of low PAH bioavailability (Wick *et al*., 2001 and 2002).

Wick and co-workers performed several studies with the anthracene-degrading *Mycobacterium* sp LB501T (Wick *et al.*, 2003*)*. They evaluated differences in growth rate and ability to degrade solid anthracene with and without addition of glucose, and they also analyzed its fatty acids and mycolic acids. They concluded that this strain degrades anthracene in the presence of glucose, however no biofilm formation was observed on anthracene when excess glucose was provided. Furthermore biofilm apparently promotes dissolution of this PAH. The substrate promoted a modification of the cell surface of the bacterium; anthracene-grown cells were more hydrophobic and more negatively charged than glucose-grown cells and could attach to the crystal surface up to 70-fold better than glucose-grown cells. Production of surfactants was not detected. Although this study was done with solid anthracene, it was still performed in liquid cultures. Thus here in this work, the study with PAH crystals on Permanox® slides was unique because there is no other report of PAH degradation using mixtures of solid PAH and analysis of bacterial communities directly from the crystals.

5 Conclusions

In the present work it was showed that artificial communities of degraders differ a lot from the intrinsic communities from the soil and sediment of which they came from. The community constructed with isolates from sediment mainly presented bacteria from known and well studied genera of degraders: *Pseudomonas*, *Bacillus* and *Mycobacterium*. On the contrary, the analysis of the biofilm formed on PAH crystals in microcosms inoculated with the sediment itself presented two genera from the phylum *Actinobacteria* (*Mycobacterium* and *Rhodococcus*) and various bacterial genera from the phylum *Proteobacteria*, but none belonging to the genus *Pseudomonas*. Like so, we concluded that unculturable bacteria play an essential role in biodegradation and they must be considered and monitored in bioremediation processes.

In the sediment community analyzed in microcosm experiment 6, the degradation processes were initially performed by strains of *alpha-, beta,* and *gammaproteobacteria* and with time strains of *Actinobacteria* (*Mycobacterium* and *Rhodococcus*) emerge and become part of the consortia. When this community was exposed to an environmental change there was a slight, but significant modification to its composition, giving rise to strains from the class of the *Deltaproteobacteria* and with the loss of bacteria from the genus *Mycobacterium*.

The classical methods for selecting degraders exclude important members of communities because they do not take into account that some strains of one community capable of degrading pollutants are not able to metabolize the compound as it is, but they are essential for the success of the consortium. We present here a simple but efficient method for selecting and recovering members of communities of PAH degraders. Although the method cannot reproduce the original consortium, it provides isolates that can form stable and active communities. Furthermore it proved to be a good method to follow the dynamics of communities composed by unculturable bacteria through culture- independent methods and is a promising technique to be used for image analyses of biofilm structure.

The method as it is presented is not yet suitable to follow the carbon flow from substrates to bacteria from an unculturable consortium by incorporation of ^{13}C in fatty acids and/or in DNA and RNA, nor to perform microarrays to map regulation of metabolic genes due to the low yield of biomass obtained on the slides. However, it is possible that using a culture of bacteria this bias may be reverted using high counts of bacteria as inoculum or working with microcosm containing larger volumes of culture.

The fact that the genus *Pseudomonas* was not abundant, and sometimes not even detected in the communities by a culture-independent method, but it was isolated through all the different approaches leads to the conclusion that this bacteria is not a main degrader in soil, but it has competitive advantages under laboratory conditions and this is reason for this genus to be often used in degradation studies.

So, to directly answer those specific key questions that I had proposed at the beginning of this thesis:

1. **Is it possible to select communities directly from the environment (without isolation of strains) capable of colonizing and degrading PAH crystals?**
   Yes, the method proposed was very effective in selecting communities of PAH degraders, which could colonize PAH crystals.
2. **How do these communities behave over time? Do their members remain over time?**
   Despite some members of the communities disappear along the time, the most abundant representatives (identified through SSCP bands) tend to remain over time.
3. **Is it possible to isolate the members of these selected communities?**
   Yes, it is possible to isolate members directly from the community attached to the PAH crystals. The strains isolated are different from those isolated through classical microbiological methods, but still they do not represent the whole community identified by culture-independent methods.

4. **Are artificial communities (those constructed with isolates) as efficient as the communities selected from the environment (without previous isolation of strains)?**

   Artificial communities are not as efficient as communities selected directly from the environment.

5. **Would different PAH substrates select for different genera of a community?**

   Yes, when different single PAH crystals are used as substrate, the resultant communities are different between each other. However, most complex HMW PAHs select for a community that are highly similar.

6. **What is the role of the genus *Pseudomonas* concerning degradation of PAHs in environmental communities?**

   Although the *Pseudomonas* genus is very well studied as a PAH degrader under laboratory conditions, this genus seems not to be as relevant in indigenous communities from the environment.

6 Perspectives and outlook

Since it was not possible to establish a very efficient community of culturable bacteria to degrade PAHs with the method utilized, a new experiment setup is proposed to select for consortia of degraders for further use in microcosm experiment.

The aim of the experiment is to create a high-throughput method for selecting a bacterial community to degrade PAHs, based on a double-tagged fluorescent *Burkholderia sartisoli* bioreporter engineered by Tecon, Binggeli and van der Meer (2009). This bacteria has the gene RP037-mChe in the chromosome, thus it is constitutively expressing and carries a plasmid containing *egfp* under control of the NAH/PHE-metabolism inducible P_{phnS} promoter by the transcription activator PhnR.

The idea is to combine different strains of bacteria in a 96 well microtiter plate of 2 ml containing minimal media and phenanthrene as a carbon source and take samples at different time points. The procedure is described below:

1. Inoculate bacterial strains into a microtiter plate containing minimal media with phenanthrene as a carbon source.
2. Sampling: Transfer the liquid culture to a new microtiter plate and centrifuge to sediment the cells.
3. Return the bacteria-free media to the original microtiter plate in the same position as it was before and keep the cells.
4. Add a culture of *Burkholderia sartisoli* sensor growth to exponential phase to the microtiter plate with the previously used media without bacteria.
5. Measure the fluorescence at different time points.

The result of the experiment will reveal the wells where the phenanthrene was more or less degraded. Wells where the bioreporter strain expresses more GFP (higher fluorescence), are those with larger amounts of phenanthrene, i.e., it was not degraded by the community. The measurement of the fluorescence should be repeated from time to time to check if the intensity of fluorescence decreases or not. If it decreases it means that all phenanthrene was consumed, if not, it means that the phenanthrene exceeded its saturation amount in the well and it is solubilised when the bioavailable fraction is consumed by the *Burkholderia*.

The bacterial community obtained from the well can be analyzed by SSCP to verify which members remained in the consortia and if there were changes during the time, then statistical modelling can be performed to unravel the influence of each strain during the degradation process.

One critical step to be carried out before construction of the communities is the selection of the isolates. SSCP gels should be cast with the candidate strains. At this point, bacteria with the same SSCP profile should be excluded. Only isolates with different running patterns should be present in one well to avoid overlapping of bands, which may prevent the elucidation of the genera present in the consortia.

Although the proposed method employs just one PAH (phenanthrene), it is rapid, easy and can be very useful as a screening procedure for bacterial communities to be tested in a microcosm experiment. A scheme of the proposed experiment is shown in Figure 42.

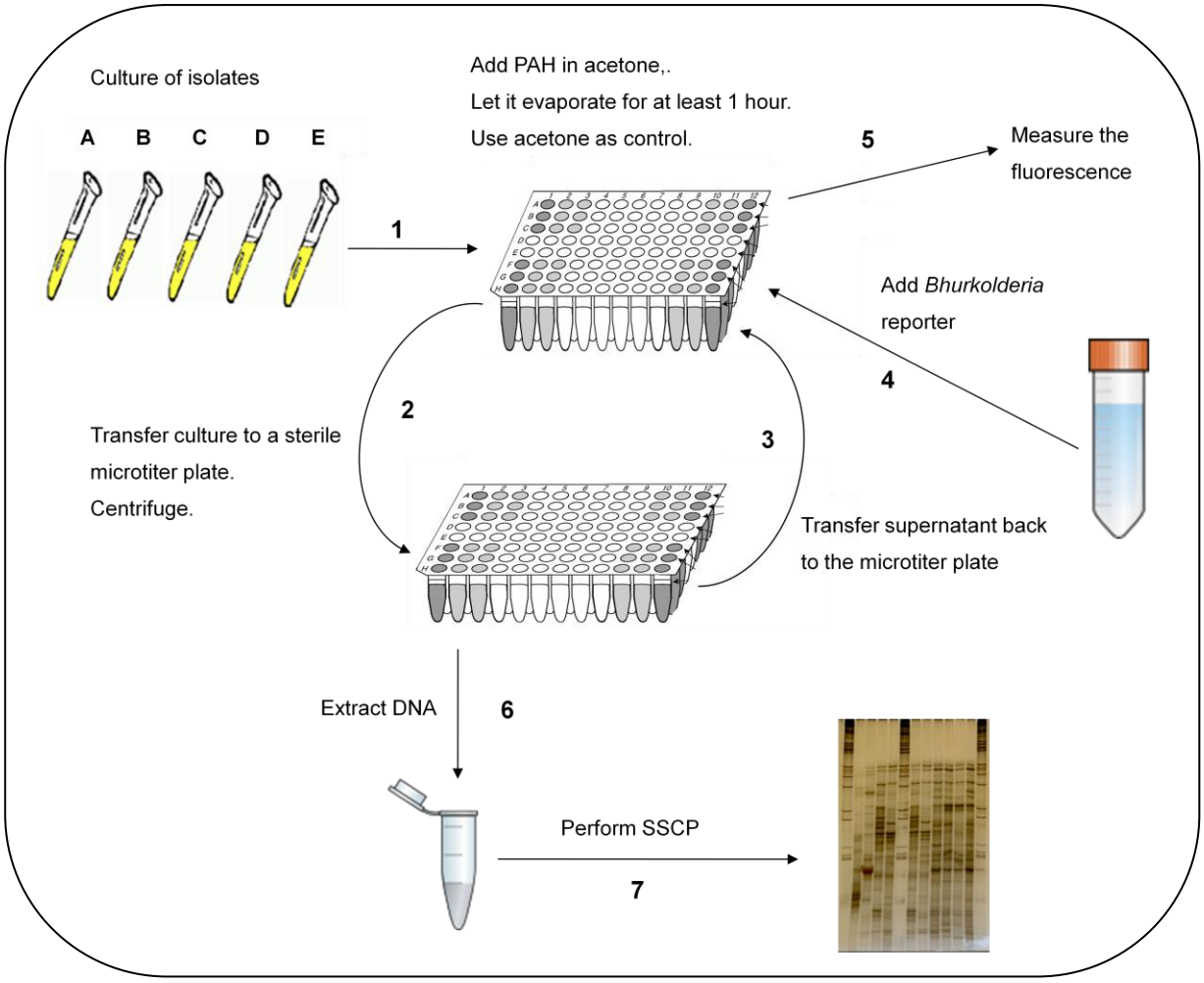


Figure 42. Scheme of the experiment proposed.

7 Supplementary Material

7.1 PCR reaction parameters

Table S 1. Composition of the 16S rRNA PCR

1 µl Forward Primer 16F27 (1 pmol end conc.)
1 µl Reverse Primer 16R1492 (1 pmol end conc.)
2 µl dNTP's (25µM each)
5 µl 10 x Taq-Buffer
38.5 µl ddH2O
2 µl Template
0.5 µl Taq-Polymerase
50 µl PCR Reaction

Table S 2. Temperature program of the 16S rRNA PCR

	Temperature	Time
	94°C	3 min
30 cycles	94°C	40 sec
	60°C	1 min
	72°C	1.5 min
	72°C	5 min

Table S 3. Composition of the Com PCR

2µl Primer Com1 (1 pmol end conc.)
2µl Primer Com2-Ph (1 pmol end conc.)
2µl $MgCl_2$
1µl dNTP's (25µM each)
5µl 10 x Taq-Buffer
35.5 µl ddH_2O
2 µl Template
0.5 µl Taq-Polymerase
50 µl PCR Reaction

Table S 4. Temperature program of the Com PCR

	Temperature	Time
	95°C	5 minutes
30 cycles	94°C	40 seconds
	50°C	40 seconds
	72°C	1 minute
	72°C	10 minutes

7.2 SSCP staining

Table S 5. SSCP staining procedure.

Bath	Composition	Time
Fixation	10% Acetic acid	30 minute
Washing	Bidistilled water	3x 5 minutes
Silver staining	0.1% Silver nitrate + 500 µL Formaldehyde	30 minutes
Washing	Bidistilled water	20 seconds
Developer	2.5% Sodium carbonate + 500 µl Formaldehyde + 500µl Sodium Thiosulfate 2%	2–5 minutes
Stop	2% Glycine + 0,5% EDTA-Na2	10 minutes
Impregnation	10% Glycerol	10 minutes

7.3 Reaction parameters for sequencing

Table S 6. Composition of the sequencing reaction

2 µl BigDye Pre-Mix (Applied Biosystems, Darmstadt)
1 µl BigDye Buffer (Applied Biosystems, Darmstadt)
2.5 µl MiliQ water
0.5 µl Primer
4 µl Template
10 µl PCR Reaction

Table S 7. Temperature program of the sequencing reaction

	Temperature	Time
	96°C	20 seconds
25 cycles	96°C	20 seconds
	60°C	20 seconds
	60°C	4 minute

7.4 Equipments

Autoclave Bioclav 32-2-3, KSG Sterilisator GmbH, Olching, Germany

Centrifuge 5415 R Eppendorf, Hamburg, Germany

Vacuum centrifuge: Concentrator 5301, Eppendorf, Hamburg, Germany

Magnetic stirrer M20/1, Framo-Gerätetechnik

Vaccum pump Aquastop, Van der Heijden, Dörentrup, Germany

Rotary shaker (flasks) Certomat BS-1, Sartorius stedim BIotech

Thermomixer compact, Eppendorf, Hamburg, Germany

Vortex Mixer Velp Scientifica

Termocycler Mastercycler Eppendorf, Hamburg, Germany

2010-001 Macrophor Electrophoresis Unit, LKB Bromma, Bromma, Sweden

Vertical Electrophoresis system, PerfectBlue Dual Gel System Twin L, PeqLab, Erlangen, Germany

HPLC Waters Alliance 2695 Separation Module, Waters 2996 Photodiode-Array-Detector and Waters 474 Scanning Fluorescence Detector (Eschborn/Germany)

NanoDrop 2000c Spectrophotometer Peglab Biotechnologie GmbH, Erlangen, Germany

Gas Chromatograph Agilent 6890N

GC-IRMS (Isotopic Ratio Mass Spectrometer) Trace Ultra, Thermo Fisher

7.5 Media, Buffer and Solutions

TE Buffer

1 M Tris-HCl pH 8.0	10 ml
0.5 M EDTA pH 8.0	2 ml
Distilled water ad	1 l

TBE Buffer (10x) (Tris-Borate-EDTA-Buffer)

Tris Base	108 g
Boric acid	55 g
$Na_2EDTA.2H_2O$	8.3 g
Distilled water ad	1 l

pH 8.0

SSCP Loading buffer

Formamide	45 %
2 M NaOH	5 mM
Bromphenol blue	0.12 %
Xylene cyanol	0.12 %

SSCP Elution buffer

Tris buffer	10 mM
KCl	5 mM
$MgCl_2.6\ H_2O$	1.5 mM
Triton X-100 pH9,0	0.1 %

PBS (Phosphate Buffered Saline)

NaH_2PO_4	0.54 g
Na_2HPO_4	0.85 g
NaCl	8.75 g
Distilled water ad	1 l

M9 medium

$Na_2HPO_4.7H_2O$	12.8 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1.0 g
Distilled water ad	1 l

pH 7.0 – 7.2

Trace elements Solution

$MgSO_4.H_2O$	4.0 mg
$ZnSO_4.7H_2O$	4.0 mg
H_3BO_4	5.0 mg
$FeCl_3.6H_2O$	2.0 mg
$H_2MoO_4.2H_2O$	1.6 mg
KI	1.0 mg
$CuSO_4$	0.4 mg
Distilled water ad	1 l

R2A medium

Yeast extract	0.5 g
Proteose peptone No.3	0.5 g
Casamini acids	0.5 g
Dextrose	0.5 g
Soluble starch	0.5 g
Sodium pyruvate	0.3 g
K_2HPO_4	0.3 g
$MgSO_4$	0.05 g
Agar	15 g
Distilled water ad	1 l

pH 7.0 $\pm$ 0.2

Bushnell Haas medium:

$MgSO_4$	0.2 g
$CaCl_2$	0.02 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
NH_4NO_3	1.0 g
$FeCl_3$	0.05 g
Distilled water ad	1 l

pH 7.0 $\pm$ 0.2

7.6 ANOVA analysis of degradation

7.6.1 Microcosm experiment 1: Waldau-Oderteich

Table S 8. ANOVA values for degradation of Fluorene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	0,284	0,01775	13,16	< 0.0001	***	Yes
Time	8	4,207	0,5259	389,8	< 0.0001	***	Yes
Column Factor	2	0,01011	0,005057	5,573	0,0237	*	Yes
Subjects (matching)	10	0,009074	0,0009074	0,6726	0,7465	Ns	No
Residual	80	0,1079	0,001349				

Table S 9. ANOVA values for degradation of Phenanthrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	0,5289	0,03306	17,09	< 0.0001	***	Yes
Time	8	4,411	0,5514	285	< 0.0001	***	Yes
Column Factor	2	0,1436	0,07182	77,88	< 0.0001	***	Yes
Subjects (matching)	10	0,009222	0,0009222	0,4767	0,9005	Ns	No
Residual	80	0,1548	0,001935				

Table S 10. ANOVA values for degradation of Anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	1,396	0,08726	19,35	< 0.0001	***	Yes
Time	8	5,945	0,7431	164,8	< 0.0001	***	Yes
Column Factor	2	0,434	0,217	57,17	< 0.0001	***	Yes
Subjects (matching)	10	0,03796	0,003796	0,842	0,5901	Ns	No
Residual	80	0,3607	0,004509				

Table S 11. ANOVA values for degradation of Fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	0,8737	0,05461	12,94	< 0.0001	***	Yes
Time	8	4,571	0,5714	135,4	< 0.0001	***	Yes
Column Factor	2	0,08558	0,04279	12,98	0,0017	**	Yes
Subjects (matching)	10	0,03296	0,003296	0,7809	0,6468	Ns	No
Residual	80	0,3377	0,004221				

Table S 12. ANOVA values for degradation of Pyrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	0,9438	0,05899	13,71	< 0.0001	***	Yes
Time	8	5,023	0,6279	146	< 0.0001	***	Yes
Column Factor	2	0,08005	0,04002	11,16	0,0028	**	Yes
Subjects (matching)	10	0,03585	0,003585	0,8334	0,598	Ns	No
Residual	80	0,3441	0,004302				

Table S 13. ANOVA values for degradation of Benzo(a)anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	1,4	0,08752	13,42	< 0.0001	***	Yes
Time	8	2,296	0,287	44,03	< 0.0001	***	Yes
Column Factor	2	0,01641	0,008205	1,102	0,3693	Ns	No
Subjects (matching)	10	0,07444	0,007444	1,142	0,3425	Ns	No
Residual	80	0,5216	0,006519				

Table S 14. ANOVA values for degradation of Chrysene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	1,033	0,06459	11,14	< 0.0001	***	Yes
Time	8	0,9888	0,1236	21,32	< 0.0001	***	Yes
Column Factor	2	0,002507	0,001254	0,2272	0,8008	Ns	No
Subjects (matching)	10	0,05519	0,005519	0,9518	0,4915	Ns	No
Residual	80	0,4638	0,005798				

Table S 15. ANOVA values for degradation of Benzo(b)fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	0,9652	0,06032	12,40	< 0.0001	***	Yes
Time	8	1,061	0,1326	27,25	< 0.0001	***	Yes
Column Factor	2	0,01188	0,005940	0,8910	0,4404	Ns	No
Subjects (matching)	10	0,06667	0,006667	1,370	0,2094	Ns	No
Residual	80	0,3893	0,004867				

Table S 16. ANOVA values for degradation of Benzo(k)fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	0,8276	0,05172	9,193	< 0.0001	***	Yes
Time	8	1,073	0,1341	23,84	< 0.0001	***	Yes
Column Factor	2	0,007368	0,003684	0,8268	0,4653	Ns	No
Subjects (matching)	10	0,04456	0,004456	0,7919	0,6365	Ns	No
Residual	80	0,4501	0,005626				

Table S 17. ANOVA values for degradation of Dibenzo(a,h)anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	3,511	0,2194	17,98	< 0.0001	***	Yes
Time	8	0,8371	0,1046	8,575	< 0.0001	***	Yes
Column Factor	2	0,4046	0,2023	12,99	0,0017	**	Yes
Subjects (matching)	10	0,1557	0,01557	1,276	0,2580	Ns	No
Residual	80	0,9763	0,01220				

Table S 18. ANOVA values for degradation of Benzo(a)pyrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	0,8633	0,05396	10,96	< 0.0001	***	Yes
Time	8	1,574	0,1967	39,95	< 0.0001	***	Yes
Column Factor	2	0,001789	0,0008946	0,2600	0,7761	Ns	No
Subjects (matching)	10	0,03441	0,003441	0,6988	0,7228	Ns	No
Residual	80	0,3939	0,004924				

Table S 19. ANOVA values for degradation of Benzo(ghi)perylene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	16	5,081	0,3175	5,128	< 0.0001	***	Yes
Time	8	2,843	0,3554	5,739	< 0.0001	***	Yes
Column Factor	2	0,3270	0,1635	3,902	0,0559	Ns	No
Subjects (matching)	10	0,4191	0,04191	0,6768	0,7427	Ns	No
Residual	80	4,954	0,06192				

Table S 20. ANOVA values for degradation of Indeno(1,2,3-cd)perylene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	14	0,6063	0,04331	4,510	< 0.0001	***	Yes
Time	7	0,7830	0,1119	11,65	< 0.0001	***	Yes
Column Factor	2	0,05551	0,02776	3,162	0,0863	Ns	No
Subjects (matching)	10	0,08778	0,008778	0,9142	0,5253	Ns	No
Residual	70	0,6722	0,009602				

7.6.2 Microcosm experiment 2: Abtsdorf

Table S 21. ANOVA values for degradation of acenaphtene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,04567	0,003806	3,425	0,0030	**	Yes
Time	3	1,836	0,6120	550,8	< 0.0001	***	Yes
Column Factor	4	0,0250	0,00625	4,688	0,0217	*	Yes
Subjects (matching)	10	0,01333	0,001333	1,200	0,3300	Ns	No
Residual	30	0,03333	0,001111				

Table S 22. ANOVA values for degradation of Fluorene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,02967	0,002472	1,854	0,0838	Ns	No
Time	3	1,915	0,6384	478,8	< 0.0001	***	Yes
Column Factor	4	0,06233	0,01558	7,792	0,0040	**	Yes
Subjects (matching)	10	0,0200	0,0020	1,500	0,1879	Ns	No
Residual	30	0,0400	0,001333				

Table S 23. ANOVA values for degradation of Phenanthrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,0530	0,004417	2,038	0,0562	Ns	No
Time	3	2,482	0,8273	381,8	< 0.0001	***	Yes
Column Factor	4	0,1377	0,03442	15,88	0,0002	***	Yes
Subjects (matching)	10	0,02167	0,002167	1,000	0,4654	Ns	No
Residual	30	0,0650	0,002167				

Table S 24. ANOVA values for degradation of Anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1523	0,01269	2,457	0,0227	*	Yes
Time	3	2,235	0,7451	144,2	< 0.0001	***	Yes
Column Factor	4	0,07167	0,01792	2,500	0,1094	Ns	No
Subjects (matching)	10	0,07167	0,007167	1,387	0,2334	Ns	No
Residual	30	0,1550	0,005167				

Table S 25. ANOVA values for degradation of Fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1637	0,01364	3,507	0,0026	**	Yes
Time	3	1,847	0,6157	158,3	< 0.0001	***	Yes
Column Factor	4	0,09233	0,02308	6,295	0,0085	**	Yes
Subjects (matching)	10	0,03667	0,003667	0,9429	0,5100	Ns	No
Residual	30	0,1167	0,003889				

Table S 26. ANOVA values for degradation of Pyrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1570	0,01308	2,201	0,0395	*	Yes
Time	3	2,227	0,7424	124,9	< 0.0001	***	Yes
Column Factor	4	0,1857	0,04642	7,527	0,0046	**	Yes
Subjects (matching)	10	0,06167	0,006167	1,037	0,4376	Ns	No
Residual	30	0,1783	0,005944				

Table S 27. ANOVA values for degradation of Benzo(a)anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1163	0,009694	1,646	0,1312	Ns	No
Time	3	0,4045	0,1348	22,90	< 0.0001	***	Yes
Column Factor	4	0,07167	0,01792	3,162	0,0637	Ns	No
Subjects (matching)	10	0,05667	0,005667	0,9623	0,4946	Ns	No
Residual	30	0,1767	0,005889				

Table S 28. ANOVA values for degradation of Chrysene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1457	0,01214	2,005	0,0605	Ns	No
Time	3	0,4752	0,1584	26,16	< 0.0001	***	Yes
Column Factor	4	0,1157	0,02892	9,132	0,0023	**	Yes
Subjects (matching)	10	0,03167	0,003167	0,5229	0,8602	Ns	No
Residual	30	0,1817	0,006056				

Table S 29. ANOVA values for degradation of Benzo(b)fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1560	0,0130	2,317	0,0307	*	Yes
Time	3	0,4232	0,1411	25,14	< 0.0001	***	Yes
Column Factor	4	0,05333	0,01333	2,963	0,0745	Ns	No
Subjects (matching)	10	0,04500	0,004500	0,8020	0,6280	Ns	No
Residual	30	0,1683	0,005611				

Table S 30. ANOVA values for degradation of Benzo(k)fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1710	0,01425	2,036	0,0565	Ns	No
Time	3	0,4365	0,1455	20,79	< 0.0001	***	Yes
Column Factor	4	0,09433	0,02358	4,162	0,0307	*	Yes
Subjects (matching)	10	0,05667	0,005667	0,8095	0,6215	Ns	No
Residual	30	0,2100	0,007000				

Table S 31. ANOVA values for degradation of Dibenzo(ah)anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,05167	0,004306	1,076	0,4127	Ns	No
Time	3	0,8258	0,2753	68,82	< 0.0001	***	Yes
Column Factor	4	0,03767	0,009417	2,018	0,1678	Ns	No
Subjects (matching)	10	0,04667	0,004667	1,167	0,3502	Ns	No
Residual	30	0,1200	0,004000				

Table S 32. ANOVA values for degradation of Benzo(a)pyrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1407	0,01172	1,758	0,1031	Ns	No
Time	3	0,4493	0,1498	22,47	< 0.0001	***	Yes
Column Factor	4	0,06733	0,01683	4,208	0,0297	*	Yes
Subjects (matching)	10	0,04000	0,004000	0,6000	0,8011	Ns	No
Residual	30	0,2000	0,006667				

Table S 33. ANOVA values for degradation of Benzo(ghi)perylene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	4,284	0,3570	8,043	< 0.0001	***	Yes
Time	3	36,82	12,27	276,5	< 0.0001	***	Yes
Column Factor	4	1,591	0,3977	14,12	0,0004	***	Yes
Subjects (matching)	10	0,2817	0,02817	0,6345	0,7727	Ns	No
Residual	30	1,332	0,04439				

Table S 34. ANOVA values for degradation of Indeno(1,2,3-cd)perylene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1020	0,008500	1,889	0,0778	Ns	No
Time	3	0,5180	0,1727	38,37	< 0.0001	***	Yes
Column Factor	4	0,05400	0,0135	3,522	0,0484	*	Yes
Subjects (matching)	10	0,03833	0,003833	0,8519	0,5852	Ns	No
Residual	30	0,1350	0,004500				

7.6.3 Microcosm 3: Microcosm W3

Table S 35. ANOVA values for degradation of Acenaphthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1978	0,01648	5,021	< 0.0001	***	Yes
Time	4	3,195	0,7987	243,3	< 0.0001	***	Yes
Column Factor	3	0,1472	0,04906	12,69	0,0010	***	Yes
Subjects (matching)	10	0,03867	0,003867	1,178	0,3340	Ns	No
Residual	40	0,1313	0,003283				

Table S 36. ANOVA values for degradation of Phenanthrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,1409	0,01174	3,530	0,0011	**	Yes
Time	4	0,6791	0,1698	51,04	< 0.0001	***	Yes
Column Factor	3	0,3471	0,1157	31,56	< 0.0001	***	Yes
Subjects (matching)	11	0,04033	0,003667	1,103	0,3820	Ns	No
Residual	44	0,1463	0,003326				

Table S 37. ANOVA values for degradation of Anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,05947	0,004956	1,635			
Time	4	0,8686	0,2171	71,66	0,1166	Ns	No
Column Factor	3	0,01253	0,004178	1,379	< 0.0001	***	Yes
Subjects (matching)	11	0,03333	0,003030	1,000	0,3005	Ns	No
Residual	44	0,1333	0,003030		0,4618	Ns	No

Table S 38. ANOVA values for degradation of Fluorene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,2345	0,01954	4,614	< 0.0001	***	Yes
Time	4	3,301	0,8251	194,8	< 0.0001	***	Yes
Column Factor	3	0,6470	0,2157	28,64	< 0.0001	***	Yes
Subjects (matching)	11	0,08283	0,007530	1,778	0,0876	Ns	No
Residual	44	0,1863	0,004235				

Table S 39. ANOVA values for degradation of Fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	12	0,07947	0,006622	1,539	0,1466	Ns	No
Time	4	0,08890	0,02222	5,165	0,0017	**	Yes
Column Factor	3	0,1569	0,05229	9,227	0,0024	**	Yes
Subjects (matching)	11	0,06233	0,005667	1,317	0,2475	Ns	No
Residual	44	0,1893	0,004303				

7.6.4 Microcosm experiment 5: Waldau 2009 – New collection

Table S 40. ANOVA values for degradation of Phenanthrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	24	5,485	0,2285	3,794	< 0.0001	***	Yes
Time	4	63,02	15,75	261,5	< 0.0001	***	Yes
Column Factor	6	6,930	1,155	15,35	< 0.0001	***	Yes
Subjects (matching)	14	1,053	0,07524	1,249	0,2683	Ns	No
Residual	56	3,373	0,06024				

Table S 41. ANOVA values for degradation of Anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	24	54,19	2,258	7,079	< 0.0001	***	Yes
Time	4	70,50	17,62	55,26	< 0.0001	***	Yes
Column Factor	6	48,49	8,081	28,33	< 0.0001	***	Yes
Subjects (matching)	14	3,993	0,2852	0,8944	0,5691	Ns	No
Residual	56	17,86	0,3189				

Table S 42. ANOVA values for degradation of Fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	24	19,70	0,8207	4,765	< 0.0001	***	Yes
Time	4	81,44	20,36	118,2	< 0.0001	***	Yes
Column Factor	6	23,12	3,854	17,11	< 0.0001	***	Yes
Subjects (matching)	14	3,153	0,2252	1,308	0,2325	Ns	No
Residual	56	9,647	0,1723				

Table S 43. ANOVA values for degradation of Pyrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	24	81,12	3,380	5,824	< 0.0001	***	Yes
Time	4	222,9	55,73	96,03	< 0.0001	***	Yes
Column Factor	6	67,46	11,24	16,78	< 0.0001	***	Yes
Subjects (matching)	14	9,381	0,6701	1,155	0,3346	Ns	No
Residual	56	32,50	0,5803				

Table S 44. ANOVA values for degradation of Chrysene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	24	42,58	1,774	3,399	< 0.0001	***	Yes
Time	4	7,571	1,893	3,626	0,0107	*	Yes
Column Factor	6	5,981	0,9968	1,443	0,2669	Ns	No
Subjects (matching)	14	9,669	0,6907	1,323	0,2237	Ns	No
Residual	56	29,23	0,5220				

7.6.5 Microcosm experiment 6: Transferred slides Waldau C

Table S 45. ANOVA values for degradation of Phenanthrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	6	0,1827	0,03044	4,449	0,0088	**	Yes
Time	3	7,844	2,615	382,1	< 0.0001	***	Yes
Sample	2	1,769	0,8844	64,14	0,0003	***	Yes
Subjects (matching)	5	0,06894	0,01379	2,015	0,1347	Ns	No
Residual	15	0,1026	0,006843				

Table S 46. ANOVA values for degradation of Anthracene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	6	4,122	0,6870	51,09	< 0.0001	***	Yes
Time	3	5,225	1,742	129,5	< 0.0001	***	Yes
Sample	2	10,18	5,091	121,7	< 0.0001	***	Yes
Subjects (matching)	5	0,2092	0,04184	3,112	0,0401	*	Yes
Residual	15	0,2017	0,01345				

Table S 47. ANOVA values for degradation of Fluoranthene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	6	0,4083	0,06806	4,017	0,0134	*	Yes
Time	3	14,07	4,689	276,8	< 0.0001	***	Yes
Samples	2	3,400	1,700	35,87	0,0011	**	Yes
Subjects (matching)	5	0,2370	0,04739	2,798	0,0559	Ns	No
Residual	15	0,2541	0,01694				

Table S 48. ANOVA values for degradation of Pyrene

Source of Variation	Df	Sum-of-squares	Mean square	F	P value	P value summary	Significant?
Interaction	6	0,2426	0,04043	1,468	0,2546	Ns	No
Time	3	11,74	3,913	142,0	< 0.0001	***	Yes
Samples	2	2,175	1,088	19,97	0,0041	**	Yes
Subjects (matching)	5	0,2722	0,05444	1,976	0,1409	Ns	No
Residual	15	0,4132	0,02755				

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Acknowledgments

I wish to thank my supervisor Dr. Wolf-Rainer Abraham for the support and for the opportunity to undertake this interesting and challenging project, the European Research Training Group “Pseudomonas: Biotechnology and Pathogenicity” for the financial support and the Helmholtz Centre for Infection Research. I also would like to thank Dr. Manfred Rohde and Dr. Thomas Neu for the microscopy images.

For accepting so kindly and promptly to review and evaluate this thesis, I thank Prof. Dr. Kenneth N. Timmis, PD. Dr.-Ing Max Schobert and Prof Dr. Dietmar Schomburg.

Many thanks to the CMIK group, where more than colleagues, I made very good friends. Special thanks for Jennifer Knaak and Esther Surges for being so kind and patient in teaching me how things worked in the lab and in Germany. I want to thank also Andréia Bergamo Estrela for the support and friendship.

I would like to include in my acknowledgments all the group “Microbial Interactions and Processes”, especially Amélia Camarinha, who always had good tips and a big smile for me and Dr. Melissa Wos-Oxley for the enormous help, without whom I would have not succeeded in writing a comprehensible thesis. Thank you so much!

I thank Marcelo Ávila de Oliveira, my partner and friend for following me overseas and helping me go through this path, sometimes very difficult, always with love and patience.

Finally, I thank all the friends from HZI that made this period of my life much smoother and fun.

Thank you all!